UNIVERSITA' DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI SCIENZE MATEMATICHE, FISICHE E NATURALI

DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA ED ECOLOGIA

CICLO XXII

HLA DQ AND ONCHOCERCIASIS IN ECUADOR: INTERACTIONS BETWEEN GENETICS AND ENVIRONMENT IN AN ENDEMIC INFECTION

Ph.D. Thesis

Flavio De Angelis

A.A. 2009/2010

Docente Guida/Supervisor: Prof. Gian Franco De Stefano

Coordinatore: Prof. Patrizia B. Albertano

INDEX

1. INTRODUCTION	**p.1**
1.1 Introduction	**p.1**
1.2 Onchocerca volvulus – life cycle and transmission	**p.4**
1.3 The disease	**p.5**
1.4 Distribution in the Americas	**p.7**
1.5 Treatment	**p.8**
1.6 APOC and OEPA	**p.9**
1.7 HLA	**p.12**
1.8 HLA class II	**p.16**
1.9 Use of SNPs as predictive markers	**p.18**
2. MATERIAL AND METHODS	**p.21**
2.1 Material and methods	**p.21**
2.2 SSO	**p.30**
2.3 SSP	**p.31**
2.4 SNP typing	**p.31**
3. RESULTS	**p.35**
3.1 Examples of the results	**p.35**
3.2 Families	**p.37**
3.3 Afroecuadorians	**p.47**
3.4 Cayapas	**p.54**
3.5 Tsachilas	**p.60**
3.6 Bamileke	**p.63**
4. DISCUSSION	**p.67**
4.1 Families	**p.67**
4.2 Population analysis	**p.70**

4.2.1 Cayapas **p.70**
4.2.2 Afroecuadorians **p.73**
4.2.3 Comparative analysis with available populations **p.79**
4.2.4 Attempt of phylogenetic analysis **p.86**

5. CONCLUDING REMARKS **p.91**

6. REFERENCES **p.93**

7. APPENDIX 1 **p.113**

1. INTRODUCTION

1.1 Introduction

Advances in human population genetics and comparative genomics have resulted in important contributions to our understanding of human genetic diversity and adaptation. For the first time, we are able to reliably detect the signature of natural selection from patterns of DNA polymorphisms. The identification of the effects of this evolutionary force provides crucial evidence to support hypotheses of human adaptation (Harris & Meyer, 2006).

This process likely occurred at two temporal levels: the first corresponds to human evolution from the time of separation from the chimpanzee common trunk to the development of anatomically modern humans (Jobling et al., 2003); at the second level, human populations under environmental pressure were subjected to micro-differentiation and adaptation to a wide variety of environmental situations.

These adaptation processes can be divided into two major categories: non-genetic and genetic. The modifications related to non-genetic adaptations like physiological, biochemical and behavioral ones are the individuals' response to changes in their actual habitats (Baker, 1988). Conversely, to demonstrate genetic adaptations, it is necessary to acquire evidence showing that natural selection has driven the evolution of particular features (Harrison, 1988).

The establishment of natural selection may be provided by different kinds and weights of evidence: we can refer to differential fertility or mortality rates associated with a particular genetic trait; *in vitro* or *in vivo* studies related to functional differences among genotypes; or even evidence of concordance in relation to the geographical distribution of some trait as a function of environmental factors that could represent a selective force (Harris & Meyer, 2006).

The actual development of comparative genomics and population genetics together with the refinement of theories of molecular evolution allowed an acceleration in the collection of new data and methodological approaches useful to effectively test assumptions related to natural selection and human adaptation, and to check specific

hypotheses concerning to the role of natural selection in shaping human genetic variation (Nielsen, 2005).

The understanding of natural selection from genetic data is not so simple: difficulty lies in the ability to detect traces of selection from the "noise" represented by the demographic history of our species. Phenomena such as expansion and/or separations and bottlenecks of course were influential in determining the genetic variability of the human gene pool up to now (Cavalli-Sforza et al., 1994).

The purpose of this PhD research is to test one of these theoretical assumptions through the study of response to infection by an environmental pathogen in two different ethnic origin communities located in marginal refuge areas of Esmeraldas province, in northwestern Ecuador.

The notion that selection during epidemics or longer periods of exposure to infectious diseases might play a major effect in modifying the constitution of the human genome is not new. It was proposed, at least in outline, in 1931: in his research, *The Inborn Factors in Disease* (Garrod, 1989), Garrod suggested that infectious diseases may have been a major selective force in human evolution and in shaping our biochemical individuality. In 1948, Haldane, (Haldane, 1948) suggested that the extremely high frequency of thalassemia in certain racial groups from the Mediterranean region might reflect an unusually high mutation rate in these populations, and he proposed that these diseases might have come under intense selection because of the heterozygote advantage against malaria. It was, in effect, Haldane's remarkable insight that opened up investigation of genetic susceptibility to infection.

Clearly, an analysis of the human genome with respect to variable susceptibility to infection is already beginning to provide important new insights into the mechanisms of human diversity.

Although less progress has been made than in the case of malaria, there is increasing evidence that variability in host responsiveness to other parasitic infections may have a strong genetic basis (Hill 1996).

Recent studies have shown that host genetics is an important determinant of the intensity of infection and morbidity due to helminthes. There is some evidence for

genetic control of filarial infection, although only few data have been reported about filarial disease. Association studies have provided evidence for major histocompatibility complex control of pathology in schistosomiasis and onchocerciasis (Hirayama et al., 1987). Recent candidate gene studies suggest a key role of other immune response genes in controlling helminth infection and pathology, but require replication. Identification of the genetic loci involved may be important in the understanding of helminth epidemiology and the mechanisms of resistance and pathology.

There have been a large number of studies of the genetic basis of susceptibility to helminth infection, principally murine studies using inbred and congenic mouse strains, or Human Leukocyte Antigen (HLA) association studies in human populations (Wakelin, 1988). However, despite the clear demonstration of genetic control in mouse models, both the importance of genetic control in humans, and the disease loci involved, had received little attention until recently.

Onchocerciasis, also known as river blindness, is caused by a nematode filarial worm that causes blindness and debilitating skin lesions. The disease occurs in 37 countries, of which 30 are in Africa, 6 in the Americas and 1 in the Arabian Peninsula. Africa is by far the most affected continent both in terms of the extent of the distribution and the severity of the clinical manifestations of the disease. Onchocerciasis remains a major parasitic endemic disease especially in Central and East Africa. The latest World Health Organization (WHO) Expert Committee on Onchocerciasis estimated that in 1995 around 17.7 million persons were infected, about 270000 of whom were blind and another 500000 severely visually impaired (WHO, 1995). Over 99% of the estimated 17.7 million persons affected with this filarial parasite live in Africa, while about 140 000 people in the Americas are infected with the worm. The disease is responsible for 1 million disability-adjusted life years (DALYs) annually. Onchocerciasis is one of the most important worldwide infectious causes of blindness, being second only to trachoma in number of affected persons (Thylefors et al., 1995; Lewalen and Courtright, 2001). Eye disease from onchocerciasis therefore represents the main public health challenge, accounting for 40% of the attributable DALYs, although severe skin disease is also

recognized as significant to public health (Hagan, 1998). Great progress has been made in the last three decades to control onchocerciasis, both in Africa and in the Americas, and this progress has been largely due to international public–private partnerships, regional programmes, sustained financing and the development of new tools and technology.

1.2 Onchocerca volvulus - Life Cycle and Transmission

The parasite, *Onchocerca volvulus*, is transmitted by small blackflies of the genus *Simulium* (Figure 1.1), which breed in fast-flowing, highly oxygenated rivers (Blacklock, 1926).

Figure 1.1 : *Simulium exiguum*

An infected blackfly deposits one or more *O. volvulus* larvae into the human host when it takes a blood meal. These larvae develop into mature adult worms in about one year. They commonly aggregate into fibrous nodules that lie under the skin, usually over bony prominences. The adult female has a mean life span of 12–15 years, with a reproductive life span of about 9 to 11 years (Habbema et al., 1992). The female is viviparous, releasing millions of embryos called microfilariae (mfs), which themselves live for about two years. The mfs that are released by the adult worm migrate from the nodules to the dermal layers throughout the body where they can be taken up by blackflies during a blood feeding act. The mfs develop into infective larvae in the *Simulium* from which they can be deposited in another host with a subsequent bite, thus completing the life cycle (Figure 1.2) of the parasite and transmitting the infection. *O. volvulus* develops only in humans and has no animal reservoir.

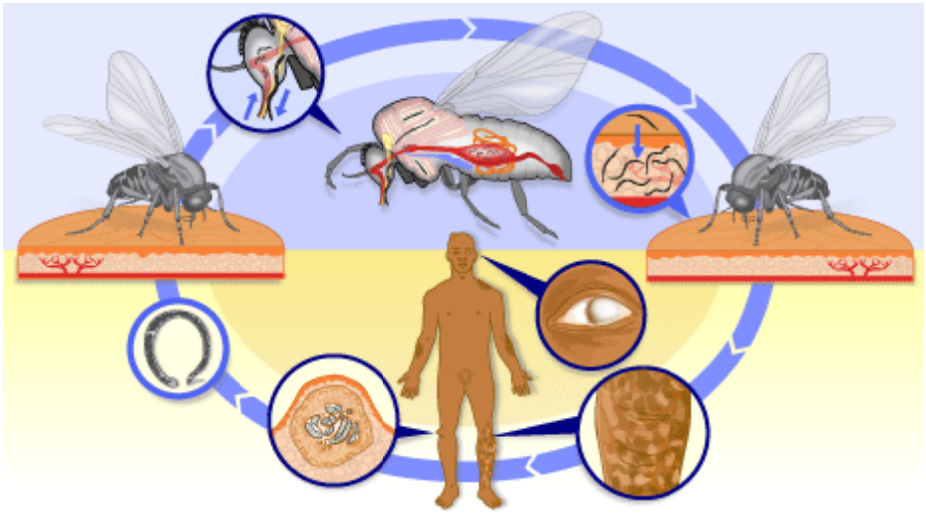


Figure 1.2: life cycle of *O. volvulus*

1.3 The disease

Clinical manifestations of onchocerciasis occur 1 to 3 years after infection, when the adult worms begin to produce mfs. Dead mfs provoke host inflammatory reactions in the tissues that present as intense skin irritation and lesions of acute papular oncho dermatitis (APOD, a solid, scattered, pruritic, papular rash. The diameter of the papules must be at least 1mm. May have vesicles or pustules at the apex and destruction of the skin crease due to edema.) (Figure1.3) and chronic papular oncho dermatitis (CPOD, a scattered, pruritic, hyperpigmented and flat-topped rash with papules that are at least 3mm.) (Figure 1.4). Severe pruritus (‘‘troublesome itching’’) is one of the most important symptoms of onchocerciasis (Brieger et al., 1998; WHO, 1995).

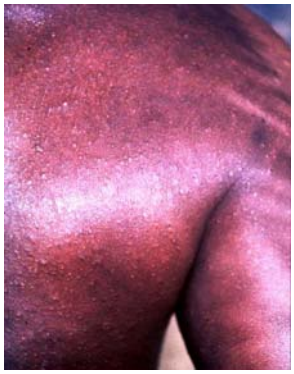


Figure 1.3 : Vesicles or pustules on the skin

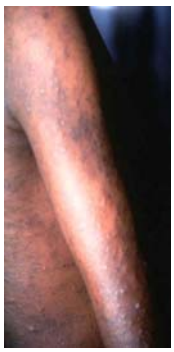


Figure 1.4 : Hyperpigmented and flat-topped rash with papules

Mfs can migrate from the skin to the eyes and cause ocular morbidity (Hall and Pearlman, 1999; Pearlman and Hall, 2000). Visual loss from acute and chronic ocular disease of both the anterior (sclerosing keratitis, irido-cyclitis) and posterior segments (optic atrophy, choroido-retinitis) occurs. In the most severe cases, blindness in one or both eyes occurs. Recent evidence suggests *Wolbachia* endobacteria (symbionts of arthropods and filarial nematodes) contain lipopolysaccharides that are released with the death of mfs and contribute to the inflammatory pathology associated with the disease (Taylor and Hoerauf, 1999; Saint Andre´ et al., 2002; Taylor et al., 2005).

1.4 Distribution in the Americas

In the Americas, onchocerciasis occurs in 13 discrete foci in six countries: Brazil, Colombia, Ecuador, Guatemala, Mexico, and Venezuela (Figure 1.5).

Figure 1.5 : American onchocerciasis foci in red

The disease was introduced into the region by the slave trade (Collins, 1992), and molecular studies indicate that it was the savanna blinding strain that was established in the Americas. In fact, the linkage between ocular disease and *O. volvulus* infection was not recognized initially in Africa, but in Guatemala by Rodolfo Robles near the turn of the last century (Kluxen & Hoerauf , 2008)

Ocular pathology attributable to onchocerciasis still occurs in many of the American foci, especially southern Venezuela, Ecuador and Brazil (Botto, 1999), but blindness is now rare to nonexistent: skin disease and pruritus are the major clinical manifestations in the region.

The foci of onchocerciasis in the Ecuadorian Province of Esmeraldas are located on Rio Cayapas and Rio Santiago (Guderian et al., 1983). There were 11 major endemic areas around Rio Cayapas and its tributaries, with an average infection rate of 51.1% of the population. The microfilarial infection rate ranged from 0% to 85% in the various regions. Based on the prevalence studies of onchocerciasis in the Province, two levels of endemicity were apparent: the upper region of Rio Cayapas with an average prevalence rate of 69.1% was found to be a hyperendemic area. All the other foci, with an average prevalence rate of 15.6% were considered hypoendemic areas (Guderian et al., 1983; Guderian et al., 1989).

1.5 Treatment

Until the 1980s only two drugs, suramin and diethylcarbamazine (DEC) were available for the treatment of onchocerciasis (WHO, 1987). Suramin is macrofilaricidal and has been successfully used in limited mass treatment (Rougemont et al., 1980, 1984), but its toxicity (damage to the kidneys) and the difficulties associated with its mode of administration (repeated injections for several weeks) have limited its usefulness for both individual and large-scale treatment (Awadzi et al., 1980 and 2003). Diethylcarbamazine (DEC) has only microfilaricidal action, has to be given over several days, and frequently produces severe adverse reactions such as fever, headache, rash, and oedema (the so-called ‘‘Mazzotti’’ reaction), especially in heavily infected people (Awadzi, 1980). DEC can also rapidly advance onchocercal eye disease leading to irreversible ocular damage. Accordingly, neither drug is recommended for routine onchocerciasis treatment or community control activities (WHO, 1995). Given the lack of an appropriate drug, which was suitable for mass treatment or field use in the 1980s, most of those infected could not be treated, and the risk of developing eye lesions and subsequently blindness and/or skin manifestations from the infection was very concrete.

Ivermectin (Mectizans, Merck & Co.) is a semisynthetic macrocyclic lactone (Figure 1.6) derived from *Streptomyces avermitilis*, which was registered for the treatment of human onchocerciasis in October 1987 in France.

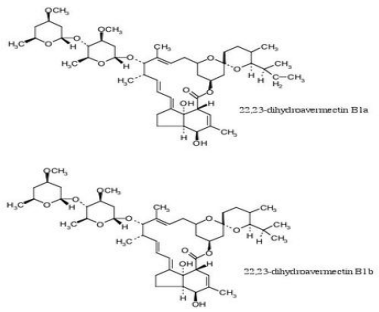


Figure 1.6 : Ivermectin formulae

Ivermectin was the first microfilaricide suitable for large-scale onchocerciasis treatment (Awadzi et al., 1985; De Sole et al., 1989; Remme et al., 1989; Prod'hon et al., 1991; Whitworth et al., 1991; Collins et al., 1992). Donated free by Merck & Co. Inc. for as long as needed, the oral medication is safe and effective when given at the standard dose of 150 mg/ kg body weight. Relative to DEC, its adverse effects are mild and non-ocular (Brown and Neu, 1990). One important characteristic is that despite its short half-life, a single dose provides long-lasting suppression of microfilaridermia (Greene et al., 1985), which makes it particularly suitable for community-based distribution in developing countries. Since the early 1990s, ivermectin has not only become the principal drug for the treatment and control of onchocerciasis, but the donation of the medicine by Merck & Co. Inc. has also opened the way for mass presumptive treatment for all those living in hyper and meso-endemic areas. A practical and easier method in which the treatment is given on the basis of height, a good surrogate for the weight of an individual (Alexander et al., 1983), has replaced the need to weigh people before treatment. This easy method allowed the distribution of ivermectin to be undertaken by communities themselves through an approach known as Community Directed Treatment with Ivermectin (CDTI). CDTI represents the philosophy of primary health care in communities to encourage responsibility for organizing their own distribution of ivermectin, which is provided to the community free of charge. Ivermectin is always available in the communities to be given at a later time to those who might have been absent, sick, or unavailable during the chosen period. Cost of delivery is therefore reduced considerably.

1.6 APOC and OEPA

Landmarks in the global control of onchocerciasis include the significant success of the Onchocerciasis Control Programme (OCP) of West Africa (1975–2002), which is held as one of the great triumphs of tropical public health, and the donation of ivermectin (Mectizans) by Merck & Co. Inc., in 1988.

Two regional programmes based primarily on ivermectin treatment are the African Programme for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination

Program for the Americas (OEPA) (WHO, 1995). The objective of APOC is similar to that of the OCP: the control of onchocerciasis to a point where it is no longer a disease of public health importance through establishment of sustainable delivery mechanisms. In the 19 APOC countries, ivermectin is distributed annually in areas where the nodule prevalence exceeds 20% (which corresponds to an mf prevalence of between 35% and 40%). The key to the evaluations of APOC is the integration of activities into the primary health care system to support distribution, as well as the continued political will and local financing for onchocerciasis control activities. The impact of the control effort is also measured in changes in the prevalence and incidence of ocular onchocerciasis and reactive onchocercal dermatitis, as well as troublesome itching (WHO, 1995).

The objective of the OEPA programme is the regional elimination of onchocerciasis, with the assumption that at some point in time ivermectin distribution will cease. WHO estimated 140 000 persons to be infected and 4.7 million to be at risk in 1995, but most at-risk population estimates are much lower now, at 500 000 persons. Over 95% of those at risk reside in Mexico, Guatemala, Ecuador and Venezuela. Studies in the Americas in the late 1980s and early 1990s using twice yearly mass treatment with ivermectin demonstrated that transmission could be interrupted in Guatemala and Ecuador. These findings helped promote a Pan American Health Organization resolution in 1991 for a coordinated regional strategy based on intensive mass ivermectin treatment in all endemic areas (including hypoendemic areas) with the objective of eliminating not only all disease manifestations but, wherever possible, transmission (Blanks et al., 1998). Key to this decision were the entomological and epidemiological characteristics of onchocerciasis in the region. Many of the American *Simulium* vector species are inefficient vectors, and sustained transmission requires high vector densities and heavy mf skin densities in infected populations. In most areas, health systems are sufficiently strong to be capable of delivering twice yearly treatments so that mf skin loads could be maintained at a level low enough to stop transmission. Both APOC and OEPA programmes use baseline and post-treatment epidemiological, entomological, and ophthalmological data from sentinel areas to measure the impact of the effort.

Differences have been described in the clinical manifestation of the disease between Africa and America (Woodruff et al., 1966). However, to date, it has not been possible to associate these differences with specific factors. The study of two ethnically well-differentiated populations who were scattered in the same extreme environment and exposed to the same selective factors may provide an opportunity to clarify this speculation. The populations involved in this research are a native indigenous population, the Cayapa Indians or Chachi, and one that traces its origin back to the 17th century African slave trade, the Afroecuadorians.

Both populations are co-resident in a marginal tropical forest area that characterizes the inner part of the province of Esmeraldas, in the northwest of Ecuador, known to be a hyperendemic zone for onchocerciasis (Guderian et al., 1989).

In Ecuador, the first evaluation of onchocerciasis was carried out in 1980 when the infection was confirmed in an Afroecuadorian male from the Province of Esmeraldas.

Several studies developed in the Rio Cayapa basin found that the prevalence and intensity of the disease were proportional to the frequency of vector/man (Guderian et al. 1988; Guderian et al., 1983).

This study also verified that the prevalence of infection among the indigenous population and that the population of African origin was virtually identical. Nevertheless, it was possible to highlight some quite important differences: first, among Cayapas, the disease seemed to show increased virulence and manifested itself with a heavier microfilaremic load than among Afroecuadorians. Beyond that, there have been significant differences in how the disease was displayed in these two populations: the dermal onchocerciasis is much more frequent in the population of African origin than in the indigenous one.

It is clear that the two populations react differently to infection, and this may be shown by the different topographical locations of the onchocercal nodules on the body surface. Among Cayapas, the nodules are predominantly located in the nuchal region and on the iliac crest, while among Afroecuadorians the distribution is mainly sacro-coccygeal.

Regarding eye pathology, in Cayapas the microfilariae are located almost exclusively in the anterior chamber, while among Afroecuadorians they are commonly found in the posterior chamber (Guderian et al., 1989).

Therefore it seems reasonable to assume that, with respect to clinical response and differential susceptibility to infection by *O. volvulus*, populations of different ethnic backgrounds may have significantly different clinical presentations. Further, these differences can be explained by particular genetic factors, such as the presence of specific alleles of HLA genes of the Major Histocompatibility Complex (MHC), as demonstrated by other parasitic diseases such as schistosomiasis (Hirayama et al., 1997) and elephantiasis (Chan et al., 1987).

1.7 HLA

For HLA we refer to a highly polymorphic region on the short arm of chromosome 6 (Figure 1.7), in a region of about one hundred genes (2-3 cM, about $4x10^6$ base pairs), while the gene for β2-microglobulin, a polypeptide chain constant binds of HLA class I, is located on chromosome 15. The HLA gene products are expressed by a variety of cells involved in the immune response to protein antigens, as the products of these genes are used to present antigens to T lymphocytes (All the HLA informations are provided by Abbas et al., 2002).

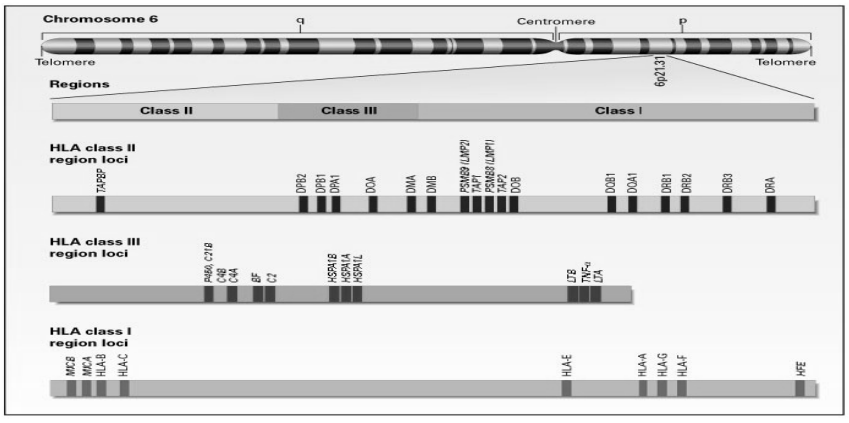


Figure 1.7 : Localization of HLA region

HLA molecules are classified as class I and class II. Each class has different structures and roles, and the association of antigens with the type of HLA (class I and II), determines the type of T lymphocytes involved in the specific immune response (CD8 + and CD4 +, respectively).

HLA class I proteins are encoded by the genes HLA-A, HLA-B, and HLA-Cw. Class I molecules are found on virtually every cell in the human body, and they present antigens to cytotoxic T-cells (CTLs) (the CD8+ T Cell). Class I molecules present "endogenous" antigens: an endogenous antigen might be fragments of viral proteins or tumor proteins (Figure 1.8).

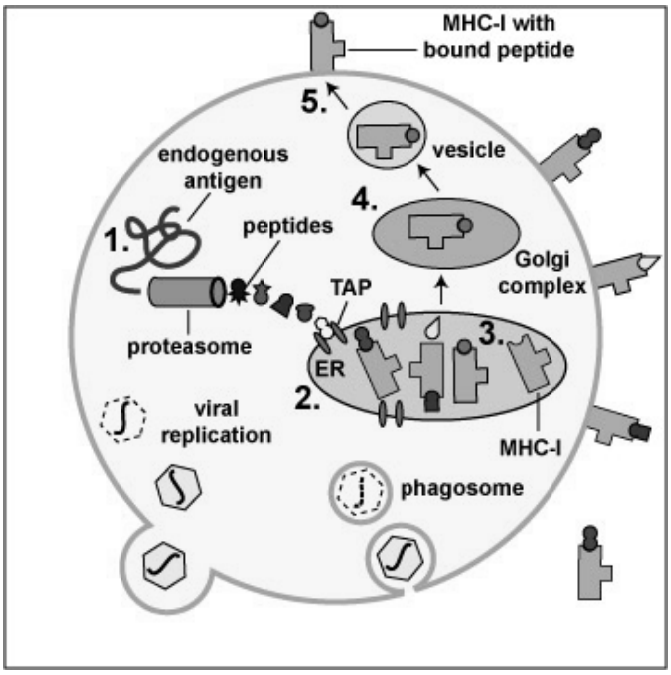


Figure 1.8 : roleof HLA class I

Presentation of such antigens would indicate internal cellular alterations that, if not contained, could spread throughout the body. Hence, destruction of these cells by CTLs is advantageous to the body as a whole. Class I molecules are made up of 2 chains, a heavy chain (transmembrane polypeptide) coded by the genes HLA-A,

HLA-B and HLA-Cw, and a light chain beta-2 microglobulin (a no-transmembrane polypeptide) (Figure 1.9).

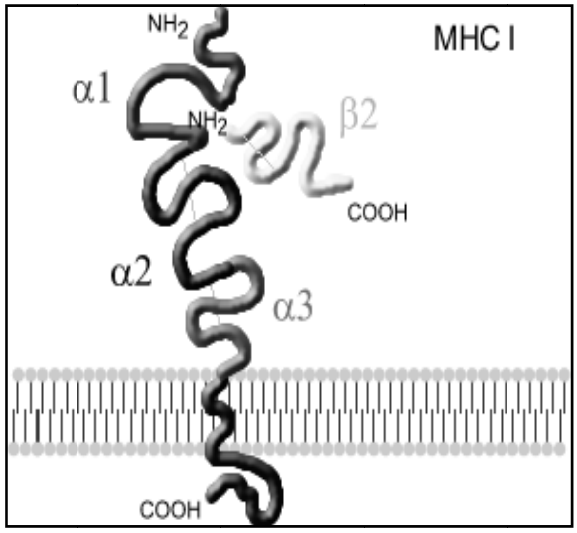


Figure 1.9 : Structure and cytological location of HLA I molecules

HLA class II proteins are coded by the gene as HLA-DR, HLA-DQ, and HLA-DP. Class II molecules, in contrast to Class I molecules, are found only in B-cells, macrophages, dendritic and other "antigen-presenting cells" (APCs). Class II molecules present antigens to helper T-cells (Th-cells) (CD4+ T cells - The CD4+ T cells that activate B cells are called Helper T cells.). Class II molecules present "exogenous" antigens. Exogenous antigens, in contrast, may be fragments of bacterial cells or viruses that are endocytosed and processed by a macrophage and then presented to helper T-cells. The Th-cells, in turn, could activate B-cells to produce antibodies that would lead to the destruction of the pathogen (Figure 1.10).

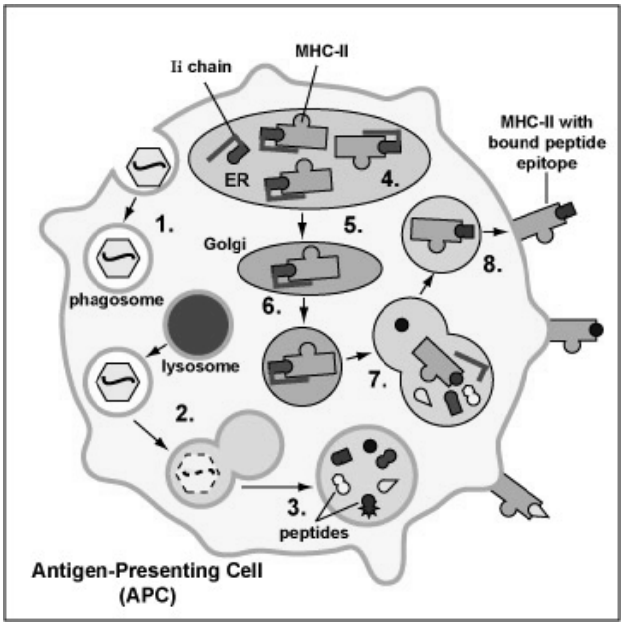


Figure 1.10 : role of HLA class II

Class II molecules consist of two transmembrane polypeptides, the Alpha chain and the Beta chain, the latter of which is much more polymorphic compared to the former (Figure 1.11).

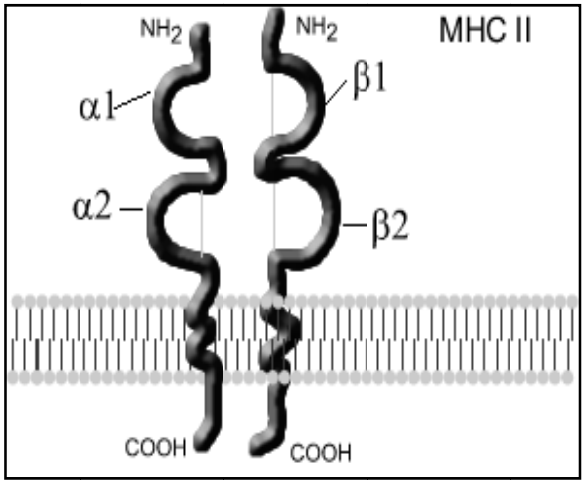


Figure 1.11: Structure and cytological location of HLA II molecules

Different allelic variants for each locus stand out in populations, each one with the capability to bind and present antigenic protein determinants: if a peptide does not bind to any MHC, T cells cannot respond to it, given that it recognizes antigens only when exposed on the surface of another cell (lymphocytes do not recognize the antigen).

Both polygenism and polymorphism give the advantage of a better chance to response with allelic combinations that may present a particular antigen in an optimal manner, so HLA can submit a lot of different peptides to T lymphocytes.

The HLA genes are co-dominant: the protein products are expressed from both chromosomes, since each individual has HLA molecules that are half paternal in origin and half maternal. In humans, certain HLA alleles or combinations are more frequent than others due to selective pressure that results in increased frequency of certain HLA alleles in specific geographic areas.

1.8 HLA class II

The HLA-D region was initially defined by the study of the mixed alloantigens lymphocyte reaction, and their identification on B lymphocytes allowed us to identify these groups using lymphotoxicity techniques. The D region contains three loci: DP, DQ, and DR (Figure 1.12).

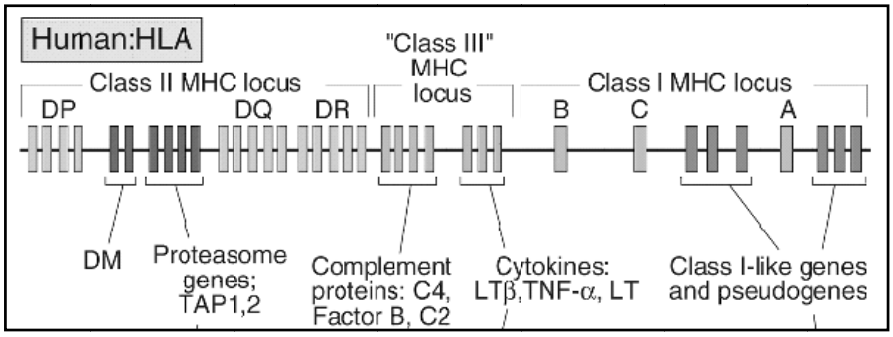


Figure 1.12 : Organization of HLA loci

D loci (DQ, DP and DR) have 5 exons: the polymorphism is mainly located at the second exon which encodes the first external domain of the polypeptide (Figure 1.13), the antigen binding domain.

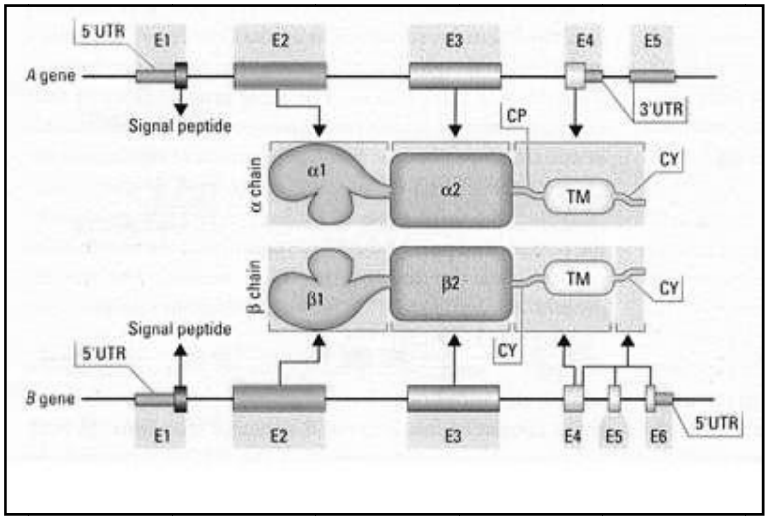


Figure 1.13 : Encoding structure of HLA class II

The series HLA-DR was the first to be described and comprises 45 DR alleles including 15 identified by serological typing (DR1-DR15), and the latter characterized by molecular biology techniques. The HLA-DQ series consists of 35 and 108 alleles respectively on the DQA locus and the DQB locus: only a few of these were recognized by serological testing. The HLA-DP series was initially described through studies of secondary mixed lymphocyte reaction between subjects with the same HLA-A, B, C, DR and DQ. This series, originally called SB, is encoded by a locus outside the HLA-DR and DQ regions. Today there are 20 DP alleles accessible to genomic typing by molecular biology.

Much research over the years has indicated the influence of HLA class II alleles in the nematode infections (Kennedy et al., 1991; Wassom et al., 1987). Recently (Meyer et al., 1994, Donfack, 1999, Murdoch, 1997), the presence of a correlation between certain allelic variants of these genes and changes in clinical manifestations of the disease caused by *O. volvulus* was highlighted. In particular, differences were found for the distribution of DQ alleles between affected and healthy individuals. The haplotype DQA1*0501-DQB1*0301 was detected significantly more frequently among immune individuals compared to patients with acute generalized or localized

disease. Conversely, the haplotypes DQA1*0101-DQB1*0501 and DQB1*0201 were more frequent in generalized disease than in healthy people.

This evidence clearly indicates that the HLA-D variants may influence infection by *O. volvulus* and that they are therefore useful to define a state that may reflect genetic protective immunity.

This research is therefore aimed at highlighting the presence of susceptibility/protective alleles in people scattered in the onchocerciasis hyperendemic focus of Esmeraldas: it may be considered the first HLA-onchocerciasis association screening in the Americas. Further, this study presents the unique opportunity to test two populations of different ethnic background co-located in a single environment, with the same selective forces, which may be very meaningful for detecting immunogenetic strength in response to onchocerciasis.

1.9 Use of SNPs as predictive markers

Recent studies examined the distribution of linkage disequilibrium (LD) along the HLA region and suggested that some SNPs (Single Nucleotide Polymorphisms) could help in the analysis of variations at HLA genes (Miretti et al., 2005, Walsh et al., 2003; Malkki et al., 2005, Simons et al., 1993).

The use of SNPs in studies on the effects of natural selection on human genes is advantageous because the use of methods for genotyping leads to the definition of a large number of identified and analyzed SNP maps (Altschuler et al., 2005 ; Hinds et al., 2005).

High levels of LD between SNPs and HLA alleles suggest that the SNPs may be informative with respect to HLA type, so carefully selected SNPs may represent a useful tool for understanding the variation in different HLA loci (de Bakker et al., 2006).

The informations provided by de Bakker and co-workers allow us to choose SNPs with the determination coefficient $r^2>0,8$ in relation to DQB1*0301, the candidate protective allele in all HLA-onchocerciasis association analyzed populations (Donfack et al.,1999; Meyer et al., 1994, Murdoch et al., 1997).

Previous data in the literature on this subject are few, so here a tagged SNP identified in the HapMap project (The International HapMap Consortium, 2005) highly correlated with HLA DQB1*0301 was analyzed. In particular, this HLA allele is associated with 2 SNPs 4 different reference populations (Africans, Chinese, Japanese and European) share the same SNPs both in the population of African origin and in the Chinese, with the allele correlated with a single SNP (rs1056315) of the previous pair in the Japanese. Thus, the genotyping of SNPs covered only the polymorphism most closely related to DQB1*0301, namely rs1056315 (Figure 1.14).

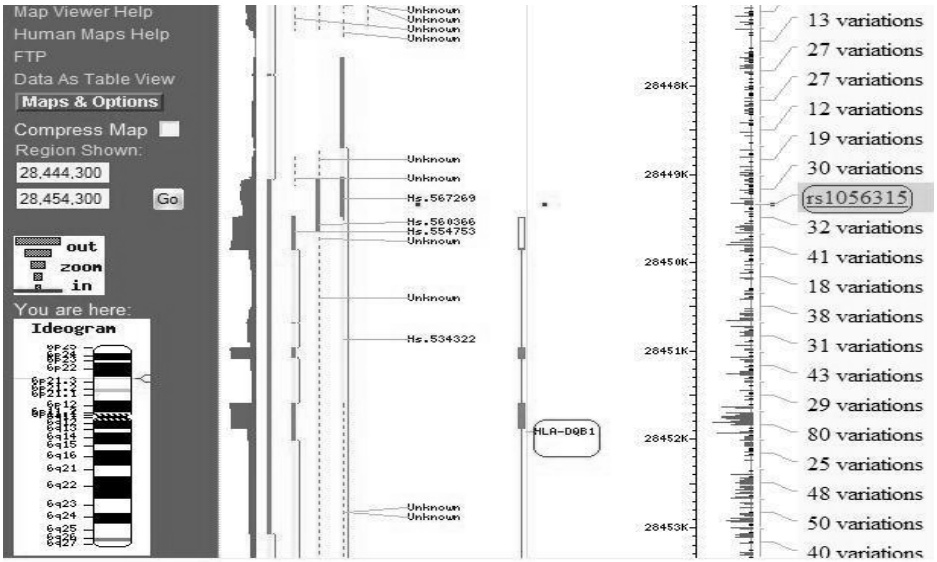


Figure 1.14 : Chromosome position of HLA DQB1 lcus and SNP rs1056315 (modified from *www.ncbi.nlm.nih.gov/pubmed/*)

2. MATERIAL AND METHODS

2.1 Material and methods

Several appropriate analyses were carried out to manage nuclear DNA to analyze HLA genes. DNA was extracted from blood samples and/or plasma (in some cases by blood coagulate) of 211 individuals of both sexes between 7 and 66 years of age, recruited in the field during the summer of 2004 (G.F. De Stefano; Dept. of Biology, University of Rome Tor Vergata). Of these, 62 subjects belong to 19 families from both communities (n = 36 Indians Cayapa, n = 26 of African origin), 74 are unrelated Cayapas, and 75 are unrelated Afroecuadorians. Through the analysis of epidemiological field data, it was possible to determine with certainty the clinical status of some sub-adult individuals, verifying by molecular diagnosis the presence/absence of microfilariae in the individual, although in low concentration.

Two apparently healthy population samples were also analyzed to compare the HLA DQ composition: 53 individuals belonging to Indios Tsachilas, an amerindian population scattered nearby the Esmeraldas Province (G.F. De Stefano) and 36 people from Bamileke community of Cameroon, kindly provided as DNA sample by G. Destro Bisol (Sapienza University, Rome).

Known as Colorados for the manner of dying their hair with a red extract from achiote (Bixa orellana), the Tsachilas currently occupy the area between the provinces of Cotopaxi and Pichincha, the canton of Santo Domingo de los Colorados. There are approximately 2600 individuals organized in communities and we analyze the community of Chiguilpe. The Colorados are, together with Cayapas, the oldest population of the territory of Ecuador (Babalini et al., 2005) .

Bamileke are an African sample scattered though the “Bamileke plateau”, in Cameroon. This population was widely investigated by G. Destro-Bisol and colleagues (Scozzari et al., 1994; Spedini et al., 1999).

The DNA was extracted from whole blood using the “Salting out” technique (Miller et al., 1988) and stored at -80°C. The DNA from plasma, extracted using the protocol of Martin and colleagues (Martin et al., 1992), gave satisfactory concentrations of nucleic acid, although the amount of DNA used for amplification

had to be greater than the standard protocols to amplify DNA extracted from whole blood (3.5 µl vs. 0.8 µl). The experimental technique is detailed in Appendix 1.

DNA extraction from samples for which it was not possible to obtain sufficient DNA concentrations was performed by coagulate based extraction using the protocol proposed by Budowle and colleagues (Budowle et al., 2000, Appendix 1):

PCR (polymerase chain reaction) is a method of DNA amplification that allows the cloning of any fragment of DNA for which the upstream and downstream regions are known (Saiki et al., 1985, Mullis et al., 1987). PCR consists of a number of cycles of denaturation and renaturation of DNA inside a special device called a "Thermal Cycler". The polymerization needs a thermostable DNA polymerase (Taq polymerase), isolated from the thermophilic bacterium *Thermus aquaticus*; a pair of oligonucleotide sequences about 20 base pairs long, called primers, bounding the region of interest; the reaction buffer; and four nucleotides. The primers are complementary to the flanking region to be amplified (template) and are oriented to allow the polymerase to synthesize in the direction of 5'-3' the new strand. Since the synthetic products are also complementary to the primers, subsequent cycles will continue to double the area of DNA between two primers. Each cycle of reaction is divided into three stages (Figure 2.1):

- Denaturation: temperature rapidly increases (from 15 seconds to 1 minute) to 94°-96°C to separate two chains that form the double helix;
- Alignment of oligonucleotides: the temperature rapidly decreases to be between 37°C and 50°C (depending on the specificity of the primers), to allow the alignment of two primers on the DNA strands;
- Extension: the temperature goes back up to reach 72°-74°C, where the efficiency of Taq polymerase is maximized, resulting in the synthesis of new strands.

By the temperature increasing to 94°-96°C, we obtain the separation of the two chains, and a new cycle can start.

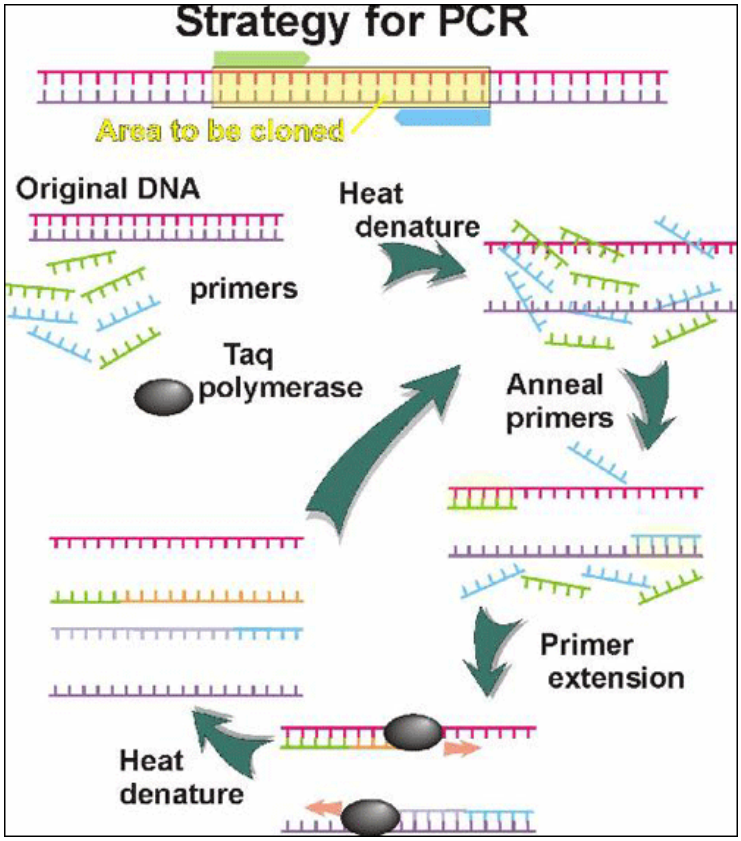


Figure 2.1 : PCR scheme

The number of cycles varies between 25 and 40, depending on the number of initial target DNA molecules (Simon et al., 1991). The final product will thus be an accumulation of templates in the order of 2^n copies, with n representing the number of cycles performed.

Electrophoresis is a series of techniques that allow the separation of any molecule, or set of molecules, with an electric charge according to different migration speeds when subjected to an electric field. These techniques involve the immersion of a three-dimensional matrix consisting of fibers of a hydrophilic polymer gel medium in an electrophoretic running buffer.

Electrophoresis gel must have the following features:

1. should not interact with the sample;
2. must be inert;
3. must be mechanically stable;
4. should allow visualization of separated molecules;
5. its "mesh" has to be checkable, i.e. the average diameter of its pores.

To optimally fit the above criteria, an excellent support for electrophoresis is agarose gel, a natural polymer that is extracted from red algae of the Gelidiacee family. Agarose forms gel because the polymer chains are joined in a rigid three-dimensional network, which keeps together the water molecules through hydrogen bridges. The polymer chains are connected via hydrogen bonds, forming double helices that are associated in groups of 10-20, and it gives rise to helical fibers that confer rigidity to the gel. To verify the successful amplification of the template by PCR, electrophoresis is performed on agarose gel using TBE buffer (0.04 M Tris Borate and 0.001 M EDTA). Five µl of each amplified product are mixed with gel loading buffer.
The gel loading buffer fits three functions:
1) increases the density of the sample, allowing the DNA to efficiently penetrate the wells of the gel;
2) colors the sample, thereby facilitating loading the amplified DNA in the gel;
3) contains a dye that migrates in an electric field towards the anode with a known speed.
The gel thus obtained is subjected to an electric field in the appropriate cell (Mini SubTM DNA Electrophoresis Cell: Bio-Rad, California, USA).
The gel is then stained with ethidium bromide and detected by exposure to UV light at 350 nm. Ethidium bromide contains a planar group that intercalates between DNA bases; therefore, the orientation and proximity of this causes the dye to increase in fluorescence compared to when it is free in solution.

The standard PCR mix is the following:

Reagents	μl
Primer F 10μM	1.5
Primer R 10μM	1.5
Buffer: ***50 mM KCl*** ***10 mM Tris-HCl pH 8.8*** ***0,1 % Triton X100***	2.5
$MgCl_2$ 15 mM	2.0
dNTPs 10 mM	1.0
Taq polymerase	0.3
ddH_2O	15.2

Sample DNA (1 µl) is added to this mix to reach 25 µl (3,5 µl of DNA extracted from plasma are required).

The oligonucleotides (primers) required to amplify the template vary according to the template itself (Table 2.1): the second and the third exons of each locus were analyzed in order to high resolution type each sample. The choice for these regions was due to their crucial role in encoding the HLA molecules (Figure 2.2): in fact these exons respectively encode for the α1 and β1 regions of the HLA DQ sub-units, which are the regions that form the antigen cleft.

Primer sequence	*Amplification*	*References*
TGTAAAACGACGGCCAG	DQB1 Exon2 fw	**Van Dijk et al., 2007**
TCGCCGCTGCAAGGTCG	DQB1 Exon2 rev	**Van Dijk et al., 2007**
GCTCACTCTCCTCTGCAA	DQB1 Exon2 rev	**Van Dijk et al., 2007**
GCTCACTCTCCTCTGCAG	DQB1 Exon2 rev	**Van Dijk et al., 2007**
TTTTCCTGTCTGTTACTGCCC	DQB1 Exon3 fwd	**Own designed**
TCAATATCCCCTTACGCCACT	DQB1 Exon3 fwd	**Own designed**
CATCTTCACTCATCAGCTGACC	DQA1 Exon2 fwd	**Cordovado et al., 2005**
GTAGAGTTGGAGCGTTTAATCAG	DQA1 Exon2 rev	**Cordovado et al., 2005**
GTAGAGTTGTAGCGTTTAATCAT	DQA1 Exon2 rev	**Cordovado et al., 2005**
GTAGAGTTGGAGCGTTTAATCAC	DQA1 Exon2 rev	**Cordovado et al., 2005**
AGGTTCCTGAGGTCACAGTGTTT	DQA1 Exon3 fwd	**Cordovado et al., 2005**
CTTGACAGACAAGAAAGCATC	DQA1 Exon 3 rev	**Cordovado et al., 2005**

Table 2.1 : Primers sequences

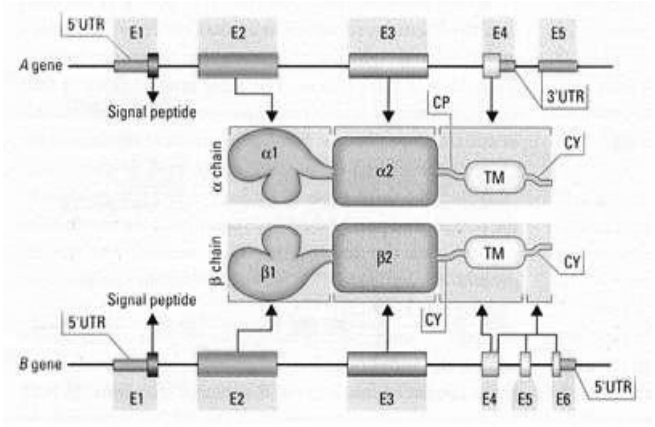


Figure 2.2 : HLA DQ encoding representation

To visualize the amplified products, 5 µl of PCR product are mixed with 1 µl of gel loading buffer, containing 0,25% xylene cyanol FF and 40% sucrose in water, and

plugged into a 2% agarose gel, before being subjected to electrophoresis and visualized under UV light at 350 nm, after staining with ethidium bromide.
The product obtained after PCR is not simply the template, but it contains a mixture of amplified sequence, primers, dNTPs (deoxynucleotide 5' triphosphate), Taq polymerase and reaction buffer. The isolation of the DNA is a crucial step before carrying on further analysis, and precipitation by ethanol according to the following protocol (Sambrook 1989) is the simplest technology to achieve pure amplified DNA (Appendix 1):
The difficulty in obtaining perfectly comprehensible electropherograms necessitated a second technique that involves the use of two enzymes such as Exonuclease I and Alkaline Phosphatase. The Exonuclease I removes single-stranded nucleic acids (primers), and alkaline phosphatase hydrolyzes the remaining dNTPs that could interfere with the sequencing reaction.
To determine the nucleotide sequence of the purified PCR products, we used the automatic sequencer ABI PRISM 3100Avant Genetic Analyzer (Applied Biosystems) located in the Department of Biology, University of Rome Tor Vergata. This machine performs a "capillary electrophoresis" in which the physical medium, which allows the separation of labeled, amplified DNA, consists of a special polymer contained in a silica capillary.
Among the many advantages offered by the application of this technique, the following can be highlighted:

- Rapidity of use, because the preparation of the gel and the loading of the sample are automatically carried out.

- Greater sensitivity, because you can get good results even from samples with low signal intensity after PCR amplification.

- The results are automatically analyzed using a special software thereby avoiding inconvenience and inconsistencies in the subjective interpretation of results.

The method is based on software that detects the fluorescence of four different colors associated with the four nucleotide bases: adenine, cytosine, thymine and guanine. In this way, every color, when it is excited by a laser, emits light at a different wavelength, and the four nucleotide bases can be easily identified. To mark the four nucleotide bases with fluorescent colors, you can use two different methods, both based on PCR, which allows the incorporation of a fluorescent compound in the template.

The first method uses 5' dye-labeled primers (dye primers), which are labeled primers in the 5' end of molecules called fluorochromes; and the second is based on the use of 3' dye-labeled dideoxy-nucleotide triphosphate (dye terminators). The choice of the method depends on the purpose of the study.

PRIMER LABELING DYE: This method enables identification of the products using primers labeled with four different fluorescent colors in four separate, base-specific reactions.

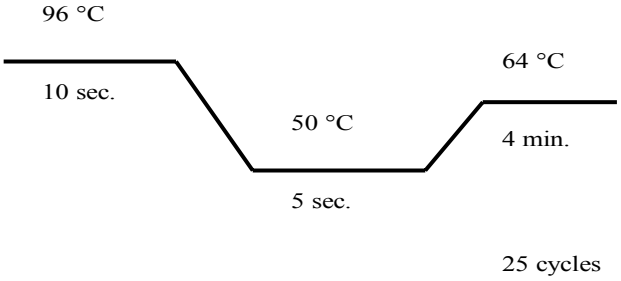
The advantages in the use of dye-labeled primers are as follows:

- Labeled primers are available for most common sites of initiation;
- They generally produce more intense signals than dye terminators.

LABELING DYE TERMINATOR: with dye terminators, each of the four dideoxy terminators (ddNTPs) is labeled with a different fluorescent color. Thus, the extension of the chain is simultaneously blocked and marked with the color that corresponds to that base. The advantages of using the dye-labeled terminators are:

- The four sequencing reactions are carried out simultaneously in a single tube;
- False stops, caused by the enzyme during the extension of the chain, can be detected by the lack of binding of dye terminators;
- The use of dye terminators requires less preparation than required with the use of dye primers.

In the present study, dye terminators were used and, in order to incorporate them into the template, a cycle was performed with the following program:



The reaction mixture (mix) contains:
1,57 μl of the primer (usually the forward amplification primer was used)
2 μl of Big-Dye (containing dye terminators, the buffer, Taq polymerase)
6,43 μl ddH_2O.

As for the PCR amplification, the marking also results in a set of labeled DNA, dye terminators, and Taq polymerase. In order to collect pure DNA, it is therefore necessary to carry out a second ethanol purification (Sambrook et al., 1989).
A second tool for the purification of marked products was used: the Centri-Sep columns. Centri-Sep columns (Princeton Separation) were used for the rapid and efficient purification of amplified labeled products by dye terminators according to the method of gel-filtration (Sambrook et al., 1989). Each unit consists of an anhydrous array contained within a special tube. This matrix is hydrated by 0,8 ml of distilled H_2O to gel after a subsequent centrifugation at 2,000 rpm for 2 minutes. The sample is loaded at the center of the gel and then subjected to further centrifugation at 2,800 rpm for 2 minutes. Once eluted, the DNA is ready for preparation for the automatic sequencer.

The preparation for the automatic sequencer consists of adding 20 μl deionized formamide to the labeled product.

Once the samples were analyzed, the raw data thus obtained were processed by the computer connected to the automatic sequencer, equipped with particular software.
The software used by the automatic sequencer is based on three processes:

- Give rise to a map made up of several peaks;
- Find the starting position and the limits of the sequence;
- Trace and extract the data obtained into a file used to read the nucleotide sequence.

This software discriminates and balances the differences in mobility and the spectral overlap between the marked bases to locate the start point and the end of a nucleotide sequence.
The output of this software is a electropherogram in which there are several peaks of different colors, each representing one nucleotide. This diagram represents the input files for other programs that allow the reading of the sequence.

2.2 SSO

A second technology was used to type HLA DQB1 locus: the Sequence Specific Oligonucleotide (SSO) hybridization. Molecular HLA typing was performed using the Inno-Lipa Multiplex and DQB1 Update kits (Innogenetics). The Inno-Lipa DQB1 Multiplex kit is designed for the amplification of the second and third exon of human leukocyte antigen DQB1 locus. The reaction is carried out using multiplex PCR format using the manufacturer's recipes. The sample to be amplified by PCR is added to a mixture of reagents containing deoxynucleotide 5' triphosphate (dNTP), biotinylated primers and thermostable DNA polymerase. In this way, we produced biotinylated target sequences. Inno-Lipa HLA-DQB1 Update is a probe test for in vitro use, designed for the molecular typing of DQB1 alleles of human leukocyte antigen.
The rationale of the test is based on reversed hybridization: amplified biotinylated DNA is denatured, and the single strands are hybridized with specific oligonucleotide probes immobilized in parallel lines on membrane strips .
This test follows a stringent washing step to remove amplified material in a non-specific binding. Alkaline phosphatase-conjugated streptavidin is added, which

binds to any biotinylated, previously formed hybrid. Incubation with a solution containing a chromogenic substrate leads to the formation of violet precipitates.
The reaction is stopped by a washing step, and the pattern of reactivity of the probes is recorded to identify which HLA DQB1 allele is present.

2.3 SSP

HLA DQA1 alleles were also typed by a different methodology: the Sequence Specific Primer (SSP) typing. Method DYNAL SSP is a PCR-based technique: it use primers that amplify specific sequences of the HLA DQA1 locus. The kit DYNAL SSP (DYNAL) consists of several pairs of allele-specific primers placed in different reaction tubes along with a mix of control and primers used to verify amplification efficiency. This technique is based on the principle that a complementary primer to a region is more efficient than primers containing one or more mismatches, so the allelic identification consists of simply determining if the amplification has been positive in a given reaction tube through visualization of the PCR product by electrophoresis.

2.4 SNP typing

The analysis of the SNP rs 1056315 was carried out by two different methodologies: direct sequencing and real time assay using LNA probes.
The direct sequencing primers and the protocol are listed below:

Primer sequence	***Primer***
AATTGAGCAGAGGCAGGAAA	**rs1056315 fwd**
GCCAGAATGTTTGAAACACCA	**rs1056315 rev**

The second methodology used is the real time assay by TaqMan® LNA probe. TaqMan probes consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end (Figure 2.3). Several different fluorophores and quenchers are available. The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source

via FRET (Fluorescence Resonance Energy Transfer). As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals.

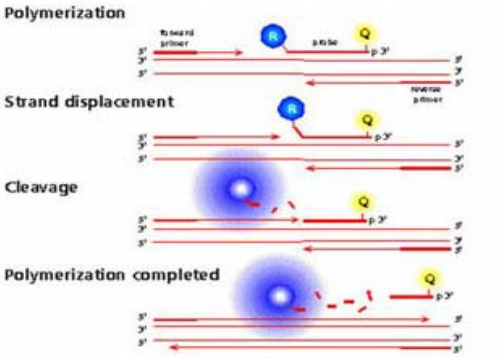


Figure 2.3 : TaqMan system

TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

The specificity of SNP detection by probe hybridization is based on the difference in melting behavior of perfectly matched duplexes with the target probe and single mismatches. The greater the difference between the melting temperature (Tm) of the matched versus mismatched duplexes, the better is the oligo probe's discriminating power. Since the stability difference between a perfectly matched duplex and a mismatched duplex can be as small as 0,5°C, the discrimination between two DNA sequences that differ by only 1 base is difficult. This Tm is dependent upon the length of the probe, the type of mismatch, and the neighboring nucleotides. The longer the probe, the smaller the effect of a single-base mismatch on overall duplex

stability. The differential stability of a matched versus mismatched duplex is the main limitation of the use of oligonucleotide hybridization for detection of SNPs.

The specificity of DNA probes can be improved by designing short probes (Thein et al., 1986); however, this is not always possible because of the Tm limitation. The Tm of short oligonucleotide probes is often significantly reduced, thus inhibiting or reducing hybridization to a minimum at the PCR annealing temperature selected for the assay.

An interesting alternative that allows the design of shorter probes for allelic discrimination was the use of probes containing LNA residues (Braasch et al., 2001). The synthesis and hybridization properties of oligonucleotides containing a novel nucleotide analog called locked nucleic acids have been reported (Kumar et al., 1998). Oligonucleotide probes containing LNA residues have shown strong affinity for their complementary targets. Because of their high thermal stability when hybridized to DNA, oligonucleotides containing LNA can be designed to have a shorter length while keeping a high Tm such that probe hybridization can occur during the PCR annealing step.

The oligonucleotides for this detection are as follows:

Primer sequence	***Oligo***	***Notes***
GATGCGACAATTGAATTATTTTC	Real time fwd	**Own designed Tm 56°C**
CACTGAAAGCATTTTGGG	Real time rev	**Own designed Tm 56°C**
cccAccAttActTcac	LNA probe T	**Own designed Tm 58** **Capitals indicate the LNA**
cccAccCttActTcac	LNA probe G	**Own designed Tm 60.5** **Capitals indicate the LNA**

3. RESULTS

3.1 Examples of the results

Molecular typing was performed using three different techniques: SSP amplification for HLA-DQA1, reverse dot blot analysis (SSO) by the Inno-Lipa kits to test HLA-DQB1, in addition to the direct sequencing of exon 2 and/or 3 to examine samples whose SSP/SSO typing did not provide acceptable results. Figures 3.1; 3.2 and 3.3 show a brief example of the typing, in order to point out the quality of the results.

Figure 3.1：Test Inno-Lipa HLA DQB1 Update

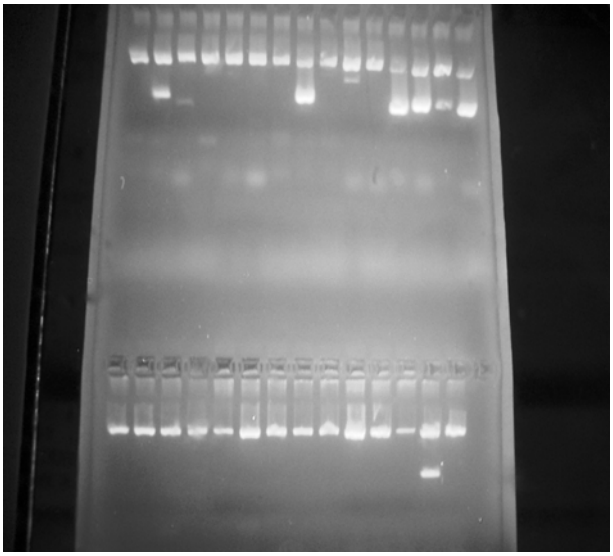


Figure 3.2 : Test Dynal SSP

Figure 3.3 : Electropherogram of direct sequencing of exon 2

The SSP data were analyzed by SSPTool, a supplied software by Dynal that detects and discriminates several electrophoresic pattern to correctly assign the HLA allele. SSO colorimetric tests were screened by a specific software like LIRAS® for LiPA HLA furnished by Innogenetics Inc.: after scanning of the LiPA strips for HLA (attached to a LiPA-*Scan* reading template), or manual entry of the probe reactivities, the LiRAS® for LiPA HLA software assisted in the interpretation of the HLA typing results. The sequence data were analyzed by direct reading and comparisons with the available HLA sequence data on *www.anthonynolan.org.uk* and the allelic assignment was confirmed by SBTengine (www.gendx.com), an user-friendly intuitive software for high-resolution HLA sequence based typing. (www.anthonynolan.org.uk and Robinson et al., 2000)

The Single Nucleotide Polymorphism (SNP) rs1056315 was detected by direct sequencing (Figure 3.4) and real time assay (Figure 3.5)

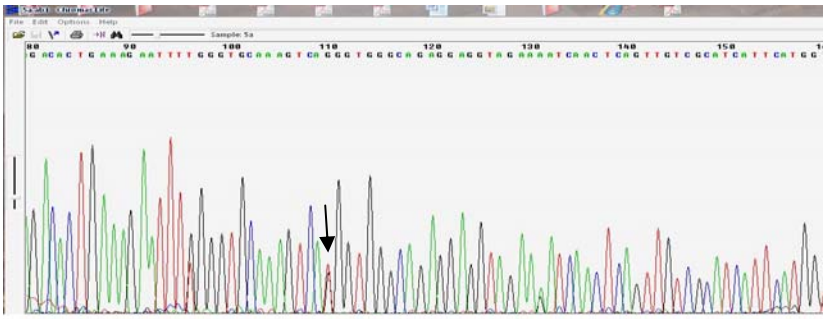


Figure 3.4 : SNP rs1056315 detected by direct sequencing

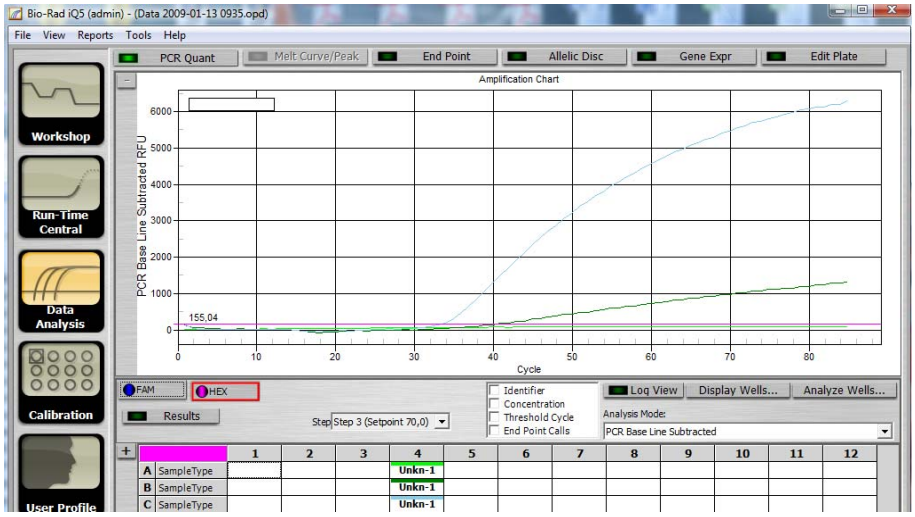


Figure 3.5 : SNP rs1056315 detected by real time assay

3.2. Families

The study of the 19 families outlines some interesting focal points regarding to the differential response to *O. volvulus* infection. The typing results are graphically expressed to quickly understand the differences between the two represented groups.

HLA DQA1	***Cayapas***	***Afroecuadorians***
0101	5	1
0102	2	19
0201		3
0202		1
0301	20	2
0302	13	5
0303		1
0401	18	8
0501	3	
0503	8	2
0505	3	10

Table 3.1 : HLA DQA1 alleles observed in the familiar samples

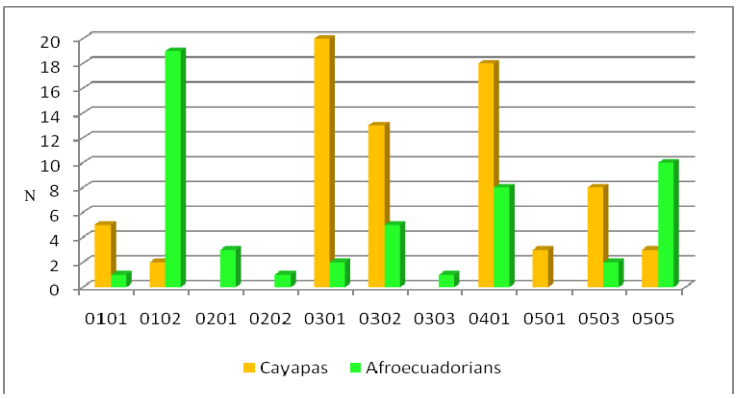


Figure 3.6 : HLA DQA1 allelic distribution observed in the familiar samples

HLA DQB1	*Cayapas*	*Afroecuadorians*
0202		3
0301	8	12
0302	23	5
0303	22	5
0402	12	5
0315	4	
0316	3	
0501		2
0502		3
0602		8
0609		6
0611		2
0619		1

Table 3.2 : HLA DQB1 alleles observed in the familiar samples

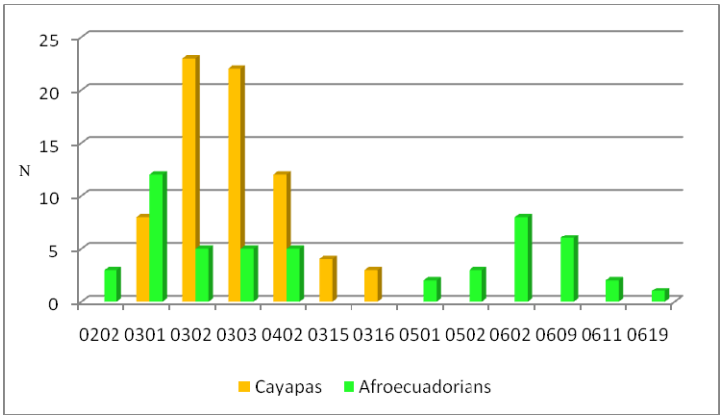


Figure 3.7 : HLA DQB1 allelic distribution observed in the familiar samples

In Tables 3.1 and 3.2 and Figures 3.6 and 3.7 the different allelic composition can be observed in the familial samples. This difference in each HLA DQ loci of course results from the different belonging of the two populations but also may reflect the pursued social model of the communities: the Cayapa Indians have always shown a strong endogamy, where weddings take place almost exclusively among individuals of the same community, and this is reflected by a limited genetic variability. Conversely the afroecuadorian community is an open community where marriages with individuals of other communities were and are very frequent: this feature allows a greater gene flow with surrounding populations, increasing genetic diversity of the community.

The samples were subdivided according to their clinical status due to onchocerciasis (Tables 3.3 and 3.4 ; Figures 3.8 and 3.9).

Cayapas HLA DQA1	*Oncho*	*No Oncho*
0101	2	3
0102	1	1
0301	12	8
0302	3	10
0401	3	15
0501		3
0503	3	5
0505	2	1

Table 3.3 : HLA DQA1 distribution in the Cayapa families according to the clinical status

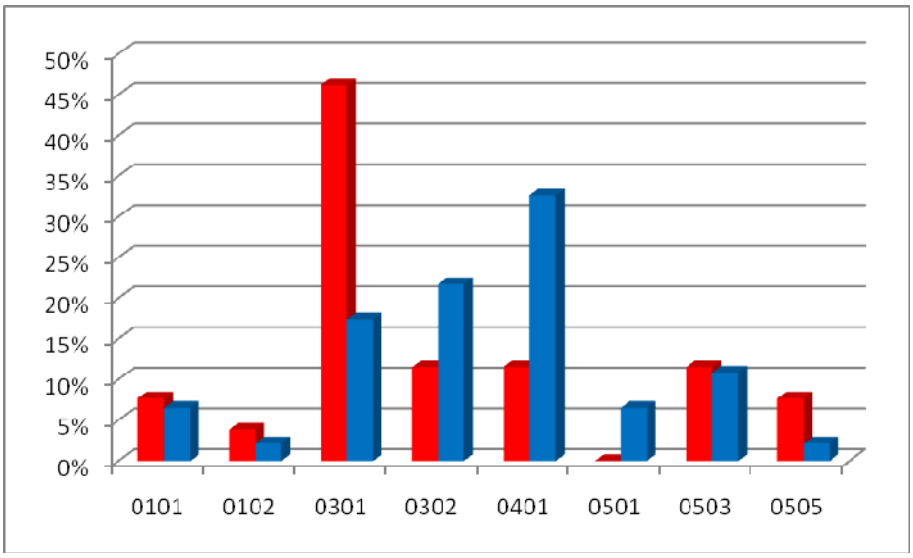


Figure 3.8 : HLA DQA1 allelic distribution in the Cayapa families according to the clinical status (red:affected individuals; blue: not affected individuals)

Cayapas HLA DQB1	*Oncho*	*No Oncho*
0301	3	5
0302	9	14
0303	10	12
0402	4	8
0315		4
0316		3

Table 3.4 : HLA DQB1 distribution in the Cayapa families according to the clinical status

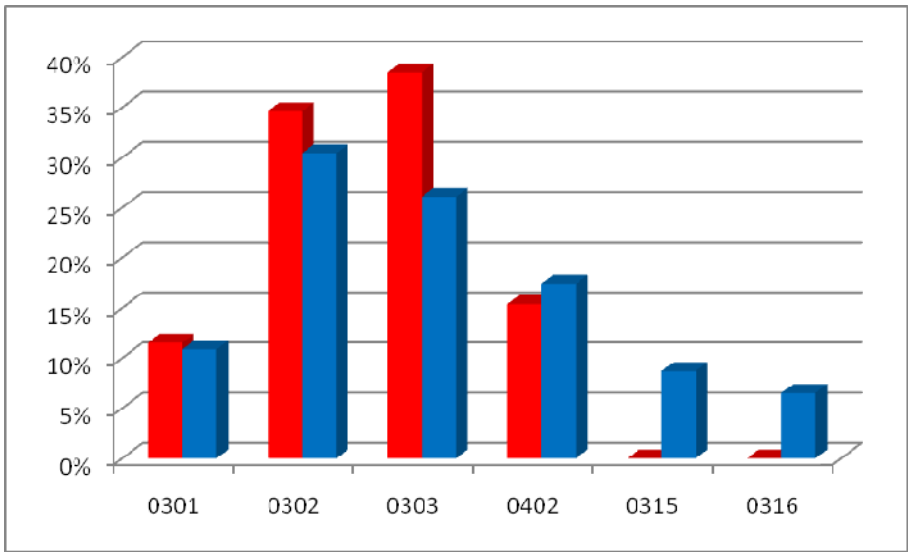


Figure 3.9 : HLA DQB1 allelic distribution in the Cayapa families according to the clinical status (red:affected individuals; blue: non affected individuals)

In Cayapas' community two alleles are significantly differently distributed between cases and controls: alleles HLA DQA1*0401 seems to be linked to healthy individuals (p=0.05) while HLA DQA1*0301 appears in strong connection with susceptibility versus onchocerciasis (p=0.01). Conversely, among DQB1 alleles, anyone seems to be selectively distributed in a single group, even if *0303 appears

to be more present in sick people than healthy ones, but the difference is not statistically meaningful.

Afroecuadorians HLA DQA1	*Oncho*	*No Oncho*
0101		1
0102	8	11
0201	1	2
0202		1
0301		2
0302	3	2
0303		1
0401	1	7
0503		2
0505	1	9

Table 3.5 : HLA DQA1 distribution in the Afroecuadorian families according to the clinical status

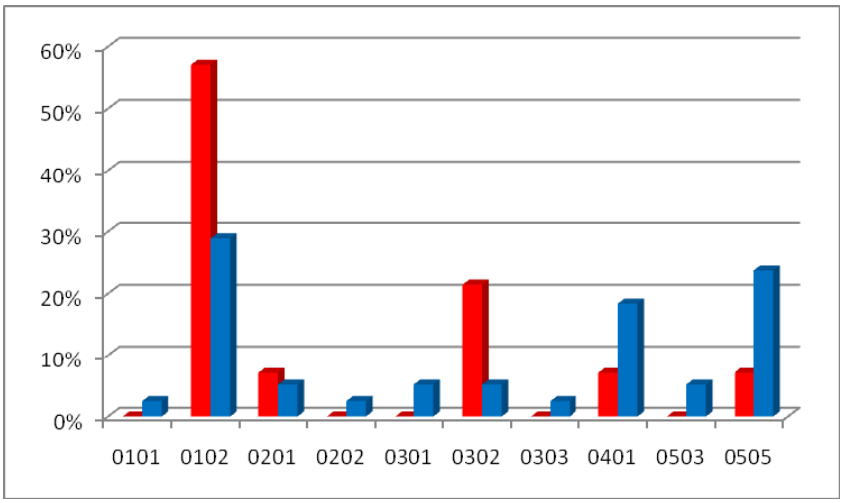


Figure 3.10 : HLA DQA1 allelic distribution in the Afroecuadorian families according to the clinical status (red:affected individuals; blue: healthy individuals)

As for the locus HLA DQA1, the afroecuadorian community shows the leading presence of two alleles in individuals supposedly protected from infection although they are represented in few subjects and for this reason they don't reach the statistical significance: HLA DQA1*0401 and HLA DQA1*0505 (Table 3.5 and Figure 3.10). These alleles do not match the supposedly protective DQA1*0501 allele, found in West Africa populations, for which we have the unique reference data (Meyer et al., 1994, Murdoch et al., 1997 and Donfack et al., 1999). Conversely, HLA DQA1*0102, without statistical significance, appears to be more present in subjects affected by onchocerciasis (57% vs 29%).

Afroecuadorians HLA DQB1	***Oncho***	***No Oncho***
0202	1	2
0301	1	11
0302		5
0303	2	3
0402	1	4
0501	1	1
0502	1	2
0602	4	4
0609	3	3
0611		2
0619		1

Table 3.6 : HLA DQB1 distribution in the Afroecuadorian families according to the clinical status

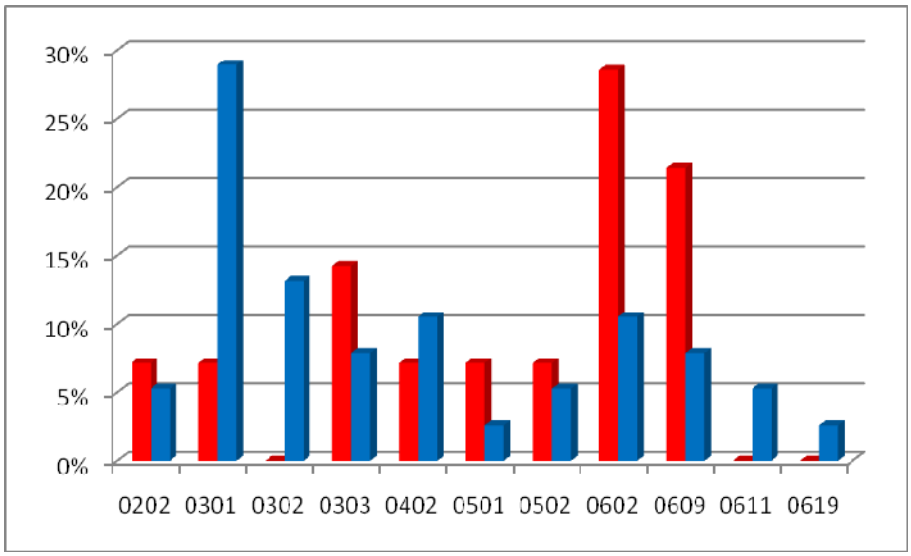


Figure 3.11 : HLA DQB1 allelic distribution in the Afroecuadorian families according to the clinical status (red:affected individuals; blue: healthy individuals)

Moreover in the afroecuadorian community seems to be a link between the HLA DQB1*0301 or *0302 and a form of protection from infection by *O. volvulus*, as expressed in Figure 3.11 and Table 3.6, but the small number of controls doesn't allow to reach significance. This result may confirm the data already present in literature (Meyer et al., 1994, Murdoch et al., 1997) but referred only to populations of the African continent.

The SNP rs1056315 genotyping allowed to classify the subjects (Table 3.7):

	Indios Cayapa		***Afroecuadorians***	
	N	%	N	%
T/T	2	5.6	7	26.9
G/T	8	22.2	5	19.2
G/G	26	72.2	14	53.9
Total	36	100	26	100

Table 3.7 : SNP rs10566315 genotypic frequencies in the Cayapa and Afroecuadorian communities

Table 3.8 shows the allele frequencies of the SNP in the samples.

	Cayapas	***Afroecuadorians***
T	0.17	0.37
G	0.83	0.63

Table 3.8 : Allele frequencies in the samples

By linking the results concerning the analysis of polymorphism rs1056315 with the molecular typing of HLA DQB1, it can be observed that the T allele of the SNP shows a moderate association with the allele DQB1*0301 in the community of African origin: in fact there is strong association between the presence of DQB1*0301 and allele T of SNP rs1056315, but not the reverse is true. In fact this can be seen by the frequencies of the estimated haplotypes between rs1056315 and HLA DQB1(Figures 3.12 and 3.13), Haplotype frequencies were computed according to Markov chain by Phase v. 2.1. (Stephens et al., 2001)

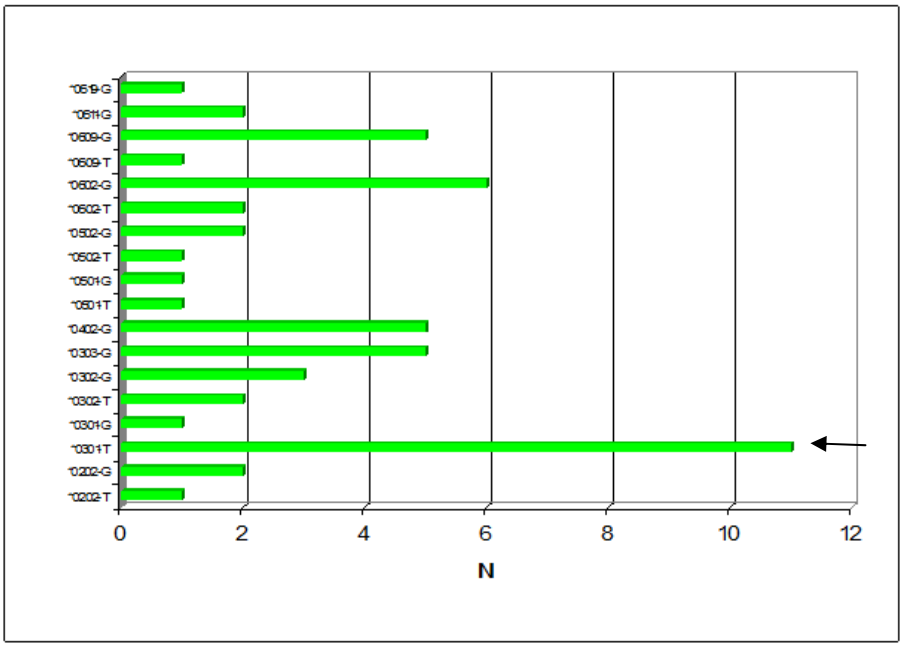


Figure 3.12 : Count of estimated haplotypes between SNP rs1056315 and HLA DQB1 in the Afroecuadorian community

This confirms, at least in part, the bibliographic data (de Bakker et al., 2006), although the correlation seems not to be very strong. In fact the frequency of haplotypes DQB1*0301-T is the leading (Figure 3.12) but also exist estimated haplotypes DQB1*0301-G and DQB1other-T, even if at low frequencies.

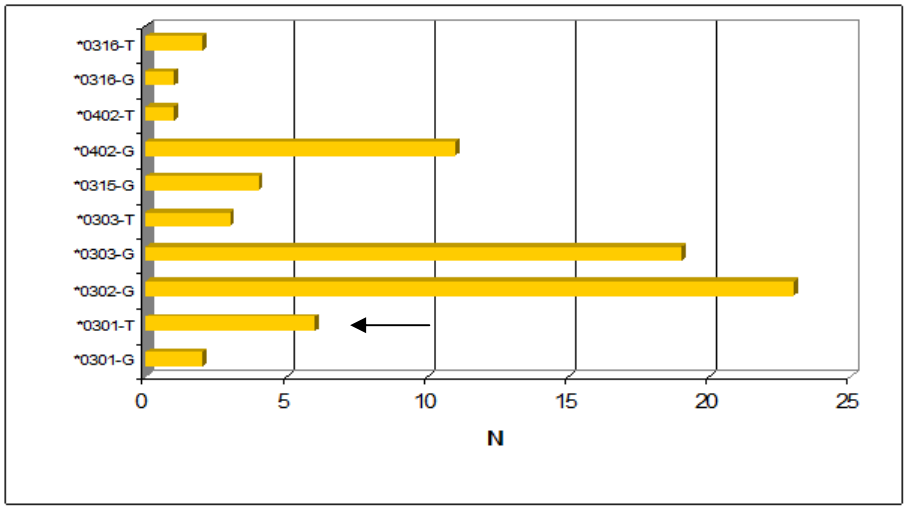


Figure 3.13 : Count of estimated haplotypes between SNP rs1056315 and HLA DQB1 in the Cayapa community

In Cayapas, conversely, this association does not seem true (Figure 3.13).

3.3 Afroecuadorians

Population sample of afroecuadorian community regards to a sample of 75 unrelated individuals. Table 3.9 and Figure 3.14 show the HLA DQA1 composition of the Esmeraldas' afroecuadorian sample. This points out an expected heterogeneity that encompass 16 variants at locus HLA DQA1, with the leading frequency of two allele, HLA DQA1*0102 and *0401, with 32% (n=48) and 29% (n=43) respectively, while the other allelic variants are somewhat less represented, with the sole exceptions of *0103 with 11%.

DQA1	*TOTAL*	*%*
0101	2	1%
0102	48	32%
0103	16	11%
0104	1	1%
0201	7	5%
0202	1	1%
0301	2	1%
0302	5	3%
0303	1	1%
0401	43	29%
0402	4	3%
0501	2	1%
0502	4	3%
0503	2	1%
0505	11	7%
0601	1	1%

Table 3.9 : HLA DQA1 distribution in the Afroecuadorian sample by percentage

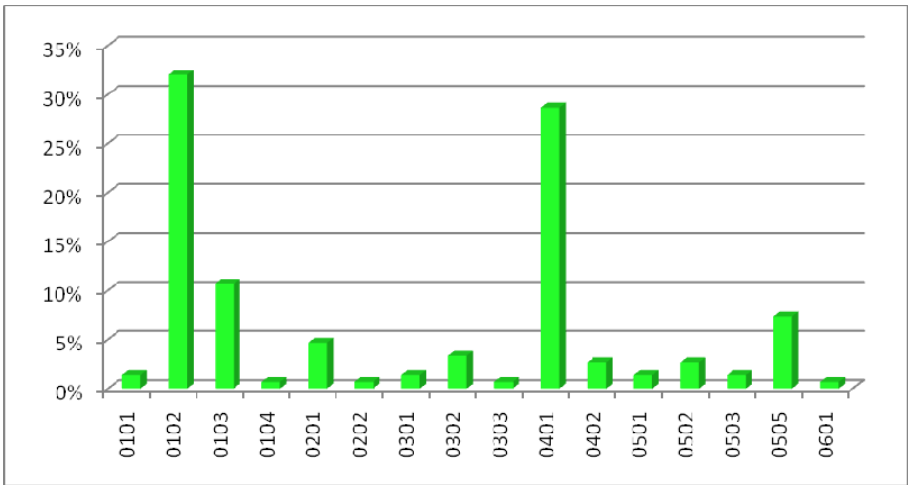


Figure 3.14 : HLA DQA1 allelic distribution in the Afroecuadorian sample

Same eterogeneity is shown by locus HLA DQB1: in fact the number of allele is quite the same but the frequencies of each is more distributed. The leading presence of DQB1*0301 (26%) is due to 39 alleles, followed by *0402 with 32 alleles (21%), and the *0611 wich reach the 10% (Table 3.10 and Figure 3.15).

DQB1	*TOTAL*	*%*
0201	5	3%
0202	1	1%
0203	1	1%
0301	39	26%
0302	4	3%
0303	10	7%
0402	32	21%
0501	8	5%
0502	2	1%
0503	1	1%
0601	2	1%
0602	14	9%
0609	7	5%
0611	15	10%
0627	9	6%

Table 3.10 : HLA DQB1 distribution in the Afroecuadorian sample by percentage

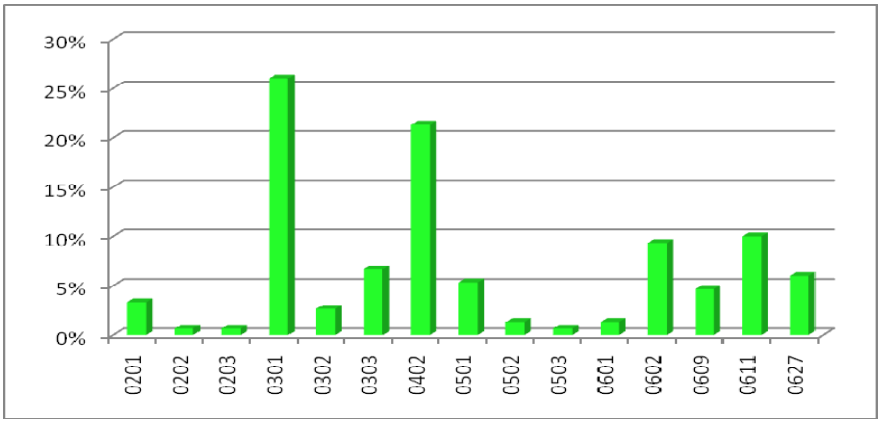


Figure 3.15 : HLA DQB1 allelic distribution in the Afroecuadorian sample

The subdivision of population according to its clinical status respect to onchocerciasis points out some interesting focal points that one could hypotize by the familial sample (Table 3.11 and Figure 3.16). In fact it is very interesting to note that DQA1*0102 confirms its susceptibility role reaching the statistical significance ($p<0.001$) along with DQA1*0103, which may be considered to be extremely statistically significant ($p<0.0001$). The same level of significance is reached by DQA1*0401 as protective allele ($p<0.0001$), also identified in familial study in a no sognificant form. Like every case/control study, Odd Ratios are calculating for each allele. The odds ratio is a measure of effect size, describing the strength of association or non-independence between two binary data values. It varies between 0 and ∞ with a threshold of 1 to indicate indipendence between variables. An odd ratio (OR) less than 1 indicates protection versus a disease, while an OR more than 1 means that the allele plays a susceptibility role in the disease. Thus the OR calculated for *0102 is 4.29 (confidence interval, CI, 2.07-8.89; $p<0.01$) and 14 was the OR for *0103 (CI 3.04-64.37; $p<0.05$): both indicate a specific susceptibility role of these alleles in onchocerciasis, and moreover the *0103 seems to be very impressive in susceptibility, but the wide confidence interval weakens the strenghtness of this role. For the HLA DQA1*0401 also was calculated the OR and the result of 0.13 (CI 0.047-0.35; $p<0.01$) confirms the protective role of this allele in onchocerciasis.

DQA1	*NO ONCHO*		*ONCHO*		*TOTAL*	
0101	2	2%	0	0%	2	1%
0102*	18	20%	30	50%	48	32%
0103**	2	2%	14	23%	16	11%
0104	0	0%	1	2%	1	1%
0201	7	8%	0	0%	7	5%
0202	1	1%	0	0%	1	1%
0301	2	2%	0	0%	2	1%
0302	1	1%	4	7%	5	3%
0303	1	1%	0	0%	1	1%
0401**	38	42%	5	8%	43	29%
0402	4	4%	0	0%	4	3%
0501	1	1%	1	2%	2	1%
0502	4	4%	0	0%	4	3%
0503	2	2%	0	0%	2	1%
0505	6	7%	5	8%	11	7%
0601	1	1%	0	0%	1	1%

Table 3.11 : HLA DQA1 distribution in the Afroecuadorian sample by percentage according to the clinical status (*p<0.01; **p<0.001)

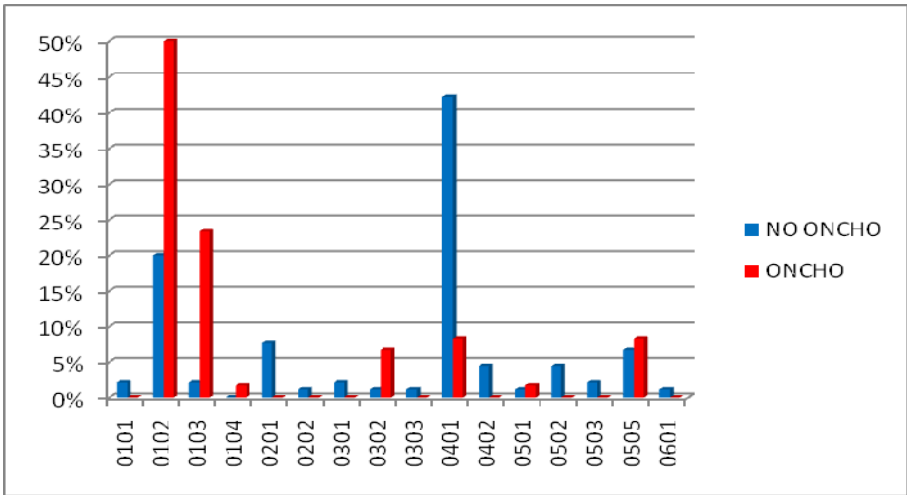


Figure 3.16 : HLA DQA1 allelic distribution in the Afroecuadorian sample according to the clinical status (red:affected individuals; blue: healthy individuals)

As for HLA DQB1 anyone allele is significantly distributed between cases and controls, even if it can be observed the leading frequency of DQB1*0301 in healthy individuals, probable heritage of their african origin (Table 3.12 and Figure 3.17). For this allele OR was estimated and the not completely significant result of 0.34 (CI 0.11-1.07; p=0.06) indicates a latent protective role, that may be weakened by the intensive gene flow occurred in this population.

DQB1	***NO ONCHO***		***ONCHO***		***TOTAL***	
0201	0	0%	5	8%	5	3%
0202	1	1%	0	0%	1	1%
0203	0	0%	1	2%	1	1%
0301	28	31%	11	18%	39	26%
0302	4	4%	0	0%	4	3%
0303	4	4%	6	10%	10	7%
0402	21	23%	11	18%	32	21%
0501	3	3%	5	8%	8	5%
0502	2	2%	0	0%	2	1%
0503	1	1%	0	0%	1	1%
0601	0	0%	2	3%	2	1%
0602	9	10%	5	8%	14	9%
0609	3	3%	4	7%	7	5%
0611	8	9%	7	12%	15	10%
0627	6	7%	3	5%	9	6%

Table 3.12 : HLA DQB1 distribution in the Afroecuadorian sample by percentage according to the clinical status

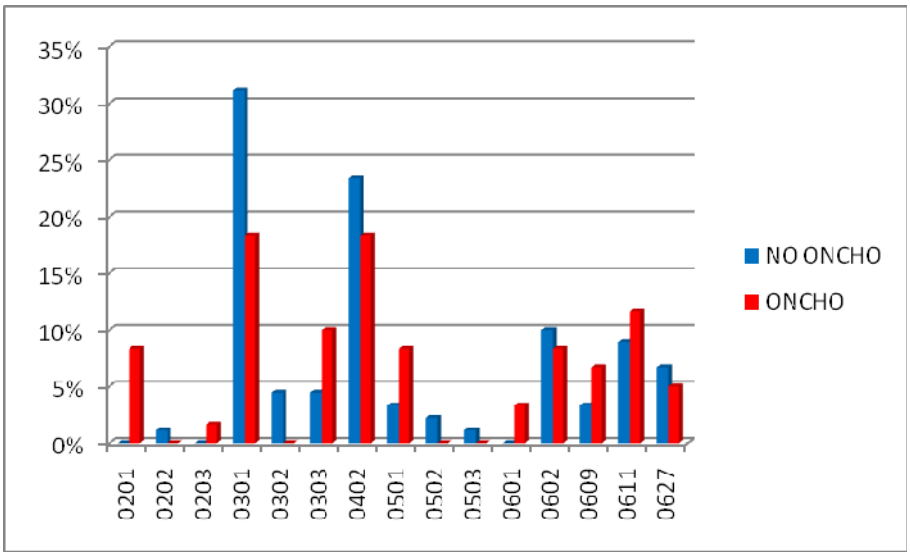


Figure 3.17 : HLA DQB1 allelic distribution in the Afroecuadorian sample according to the clinical status (red:affected individuals; blue: healthy individuals)

The sample was typed for rs1056315 and the result of this is shown in Table 3.13

rs1056315			
	N		***%***
G/G	48	**G**	73
G/T	13	**T**	27
T/T	14		
TOTAL	75		100

Table 3.13 : Genotypic and allele frequencies of SNP rs1056315

To investigate the potential predictive role of this SNP for HLA DQB1*0301, haplotypes were estimated by Phase v.2.1 (Stephens et al., 2001).

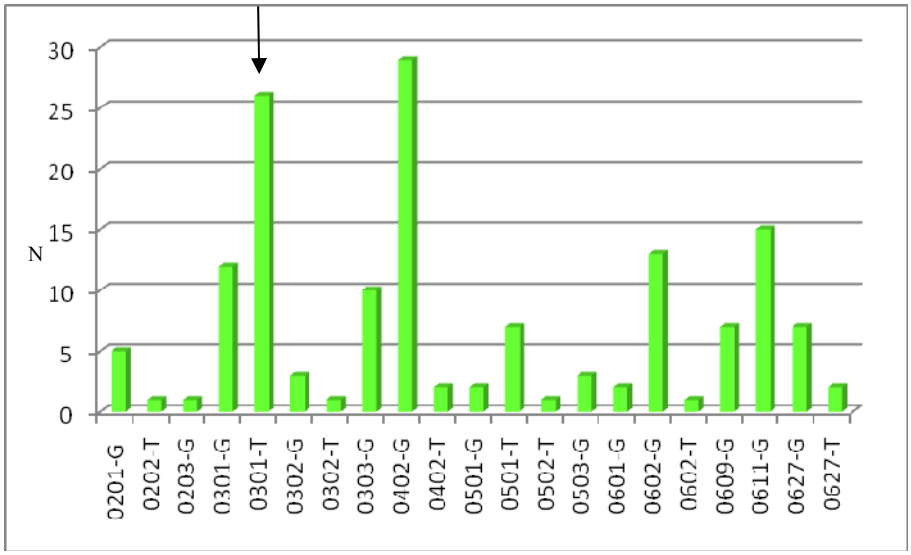


Figure 3.18 : Esimated haplotype distribution between HLA DQB1 and SNP rs1056315 in the Afroecuadorian community

As showed in Figure 3.18 there is a leading frequence of the haplotype DQB1*0301-rs1056315T that occours in 26 genomes while the others HLA DQB1-rs1056315T haplotypes do not reach the 8 genomes. More distributed is the G allele that is in connection with all the HLA DQB1 alleles.

3.4 Cayapas

Population sample of Cayapa Indians involves 74 unrelated individuals. Table 3.14 and Figure 3.19 point out the HLA DQA1 allelic pool. This encompasses 11 allelic variants with the foremost frequency of three alleles: DQA1*0301, *0302 and *0401 (respectively 30%, 18% and 24%); with the rest in somewhat low frequencies.

DQA1	*TOTAL*	*%*
0101	6	4%
0102	4	3%
0301	44	30%
0302	26	18%
0401	35	24%
0402	6	4%
0501	7	5%
0502	6	4%
0503	8	5%
0505	4	3%
0601	2	1%

Table 3.14 : HLA DQA1 allelic pool in the Cayapas

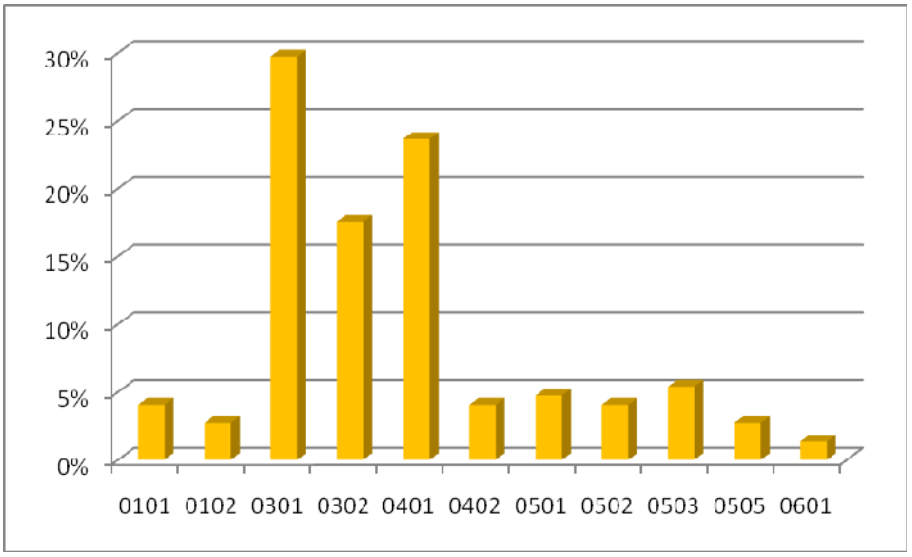


Figure 3.19 : HLA DQA1 allelic distribution in the Cayapas

As for locus HLA DQB1, Cayapas shows 10 alleles with the leading frequencies of alleles *0301 (14%), *0302 (24%), 0303 (24%) and *0402(22%). The *03 group encompasses the majority of the variation with the 72% of the entire allelic pool (Table 3.15 and Figure 3.20).

DQB1	*TOTAL*	*%*
0201	3	2%
0301	20	14%
0302	46	31%
0303	36	24%
0315	5	3%
0316	4	3%
0317	1	1%
0402	32	22%
0602	1	1%

Table 3.15 : HLA DQB1 allelic pool in the Cayapas

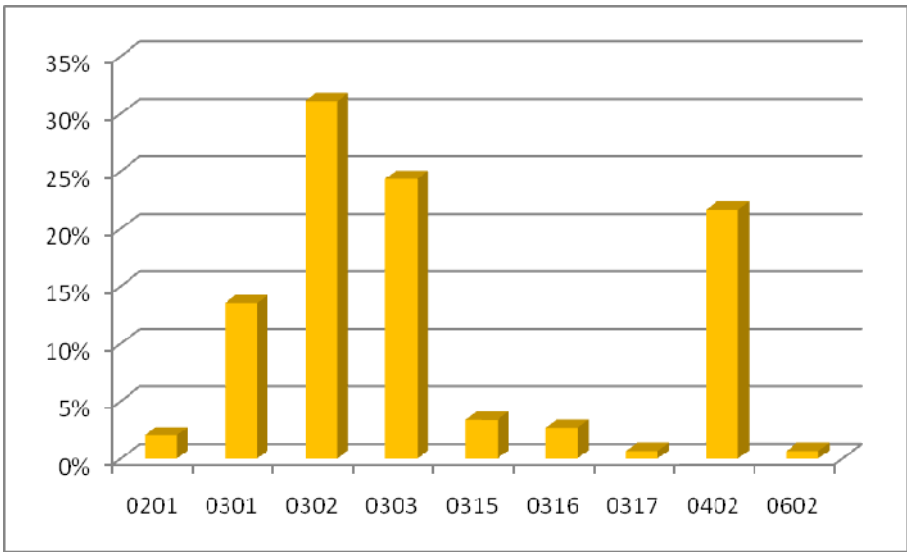


Figure 3.20 : HLA DQB1 allelic pool in the Cayapas

The population was subdivided into two classes according to presence/absence of onchocerciasis features and some interesting findings can be outlined by observation of the differences (Table 3.16 and Figure 3.21)

DQA1	*NO ONCHO*	*%*	*ONCHO*	*%*
0101	3	4%	3	4%
0102	2	3%	2	3%
0301*	14	21%	30	38%
0302	14	21%	12	15%
0401**	24	35%	11	14%
0402	1	1%	5	6%
0501	4	6%	3	4%
0502	0	0%	6	8%
0503	3	4%	5	6%
0505	1	1%	3	4%
0601	2	3%	0	0%

Table 3.16 : HLA DQA1 allelic pool in the Cayapas divided according to the clinical status (*p<0.05; **p<0.01)

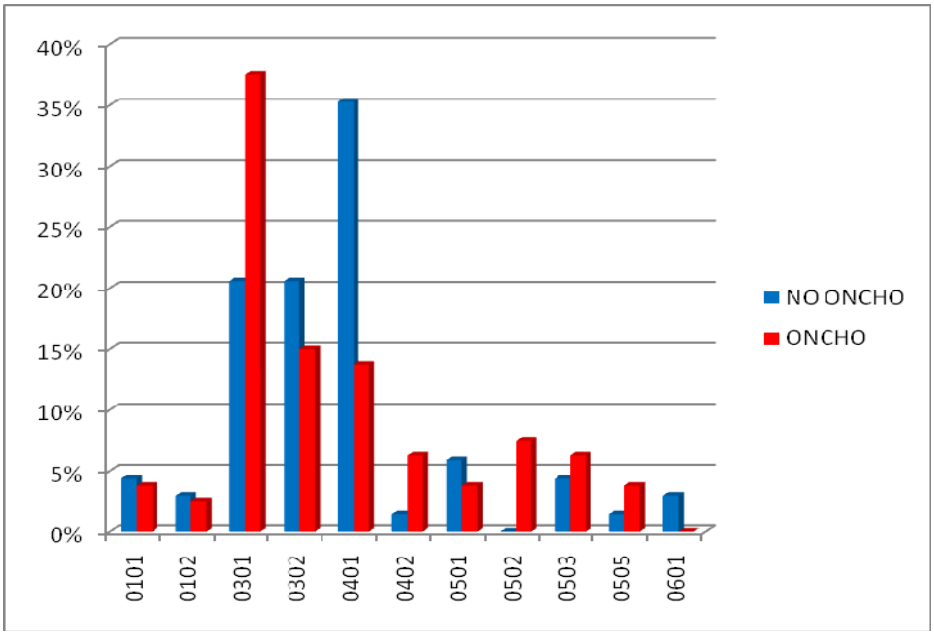


Figure 3.21 : HLA DQA1 allelic pool in the Cayapas divided according to the clinical status

In fact, as previewed in the familial sample, the allele HLA DQA1*0401 seems to represent a significant protective allele: its frequencies between healty and affected individuals are extremely significantly dissimilar ($p<0.01$). Conversely the allele DQA1*0301 appears to be a true susceptibility variant among HLA DQA1 locus, with a leading frequence in affected individuals ($p<0.05$). Odd ratios are estimated for these loci and the results confirm the hypotesis provided. OR for DQA1*0301 indicates a moderate susceptibility effect of this variant (OR 2.31, CI 1.01-4.09; $p<0.05$); while allele *0401 confirms to be associated to a strong protective status with an OR = 0.29, CI 0.13-0.66, $p<0.01$).

For locus HLA DQB1 (Table 3.17 and Figure 3.22), the only allele able to reach the statistical significance is the DQB1*0402, which meaningfully appears in healthy people ($p<0.05$) and for which OR=0.42 (CI 0.19-0.95; $p<0.05$) could confirm the protective status. HLA DQB1*0302 also seems to be not randomly distributed between groups, although its major frequency in affected people does not reach the statistical significance (OR=1.95; CI 0.95-4; $p>0.05$).

DQB1	***NO ONCHO***	***%***	***ONCHO***	***%***
0201	0	0%	3	4%
0301	8	12%	12	15%
0302	16	24%	30	38%
0303	17	25%	19	24%
0315	4	6%	1	1%
0316	2	3%	2	3%
0317	0	0%	1	1%
0402*	20	29%	12	15%
0602	1	1%	0	0%

Table 3.17 : HLA DQB1 allelic pool in the Cayapas divided according to the clinical status

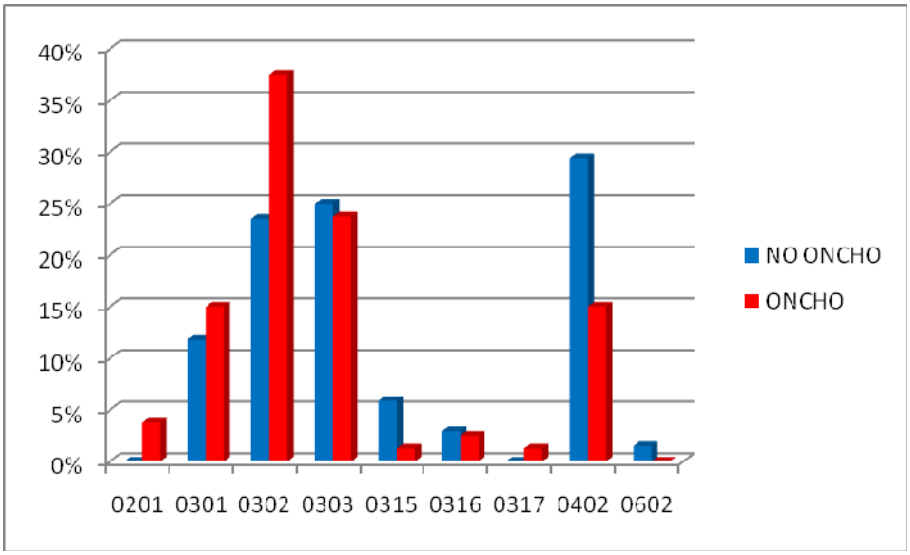


Figure 3.22 : HLA DQB1 allelic distribution of the Cayapas according to the clinical status

Like the afroecuadorian sample, the Cayapas was typed for rs1056315, in order to investigate the potential predictive role of the T allele of this SNP in inferring the presence of HLA DQB1*0301. Table 3.18 points out the result of the typing in the sampled population.

rs1056315			
	N		**%**
G/G	56	**G**	83
G/T	11	**T**	17
T/T	7		
	74		100

Table 3.18 : Genotypic and allele frequencies of rs1056315 in the Cayapa's sample

Figure 3.23 shows the estimated haplotypes for the couple HLA DQB1-rs1056315, and it can be easly detected the lack of association between T allele of the SNP and the selected HLA variants. In fact the T allele is not quite represented in sample and it doesn't exceed the 10 haplotypes, with the correlation coefficient $r^2 = 0.026$.

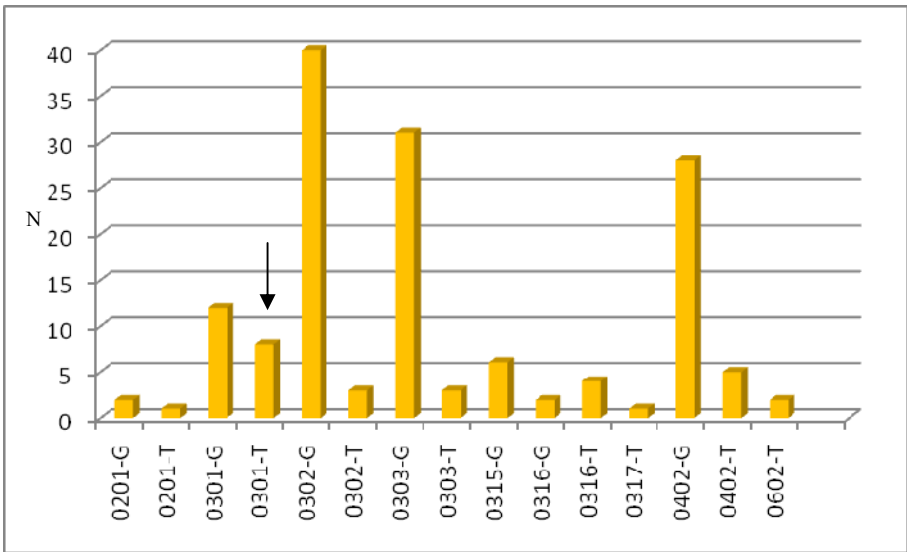


Figure 3.23 : Estimated haplotype distribution between HLA DQB1 and SNP rs1056315 in the Cayapa's community

3.5 Tsachilas

Tsachilas' sample was molecularly typed for HLA DQA1 and HLA DQB1 in order to allow a comparison between Esmeraldas samples and human group near scattered in north-western Ecuador. This samples did not be recruited for a case/control study, thus it is impossible to classify the individuals according to their clinical status regarding to onchocerciasis.

As for HLA DQA1 in Tsachilas, 11 allelic variants are found, with three alleles that reach appreciable frequencies: *0301 with 16 alleles (15%), *0401 with 30 alleles (29%) and 29*0501 (28%) (Table 3.19 and Figure 3.24).

DQA1	*TOTAL*	*%*
0102	4	4%
0201	5	5%
0301	16	15%
0302	3	3%
0303	2	2%
0401	30	29%
0402	5	5%
0501	29	28%
0502	3	3%
0503	1	1%
0601	6	6%

Table 3.19 : HLA DQA1 allelic pool in the Tsachilas community

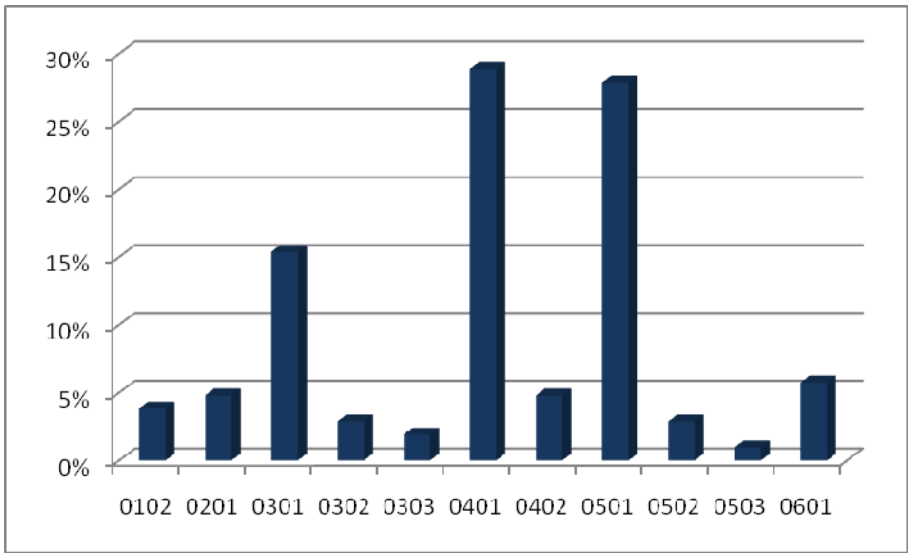


Figure 3.24 : HLA DQA1 allelic distribution in the Tsachilas

The DQB1 allelic pool of Tsachilas encompasses 10 variants: 3 alleles seem to reach frequencies more than 10%: DQB1*0201 (16%), *0301 (20%) and *0302 (23%) (Table 3.20 and Figure 3.25).

DQB1	*TOTAL*	*%*
0201	17	16%
0202	6	6%
0301	21	20%
0302	24	23%
0303	9	9%
0304	2	2%
0401	8	8%
0402	2	2%
0501	8	8%
0502	7	7%

Table 3.20 : HLA DQB1 allelic pool in the Tsachilas

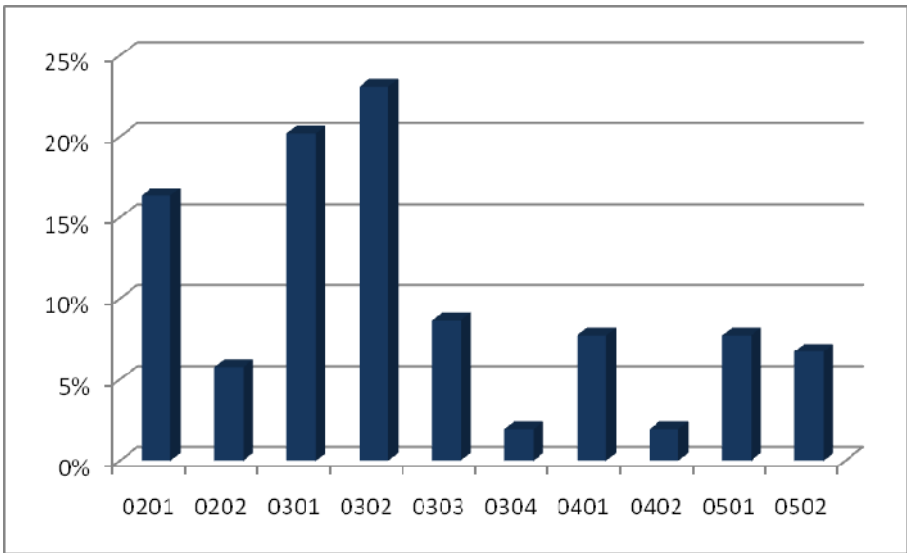


Figure 3.25 : HLA DQB1 allelic distribution in the Tsachilas

3.6 Bamileke

The African sample of Bamileke was molecularly typed for HLA DQ loci, in order to represent a face off to Esmeraldas Afroecuadorians. As it can be expected, the variation at HLA DQ loci is quite more than American samples, in fact DQA1 allelic pool is represented by 14 alleles, with distributed allelic frequencies in every class, ranging from 17% of *0102 to 1% of *0101, *0104 and *0504 (Table 3.21 and Figure 3.26).

DQA1	*TOTAL*	*%*
0101	1	1%
0102	12	17%
0104	1	1%
0201	7	10%
0301	3	4%
0302	4	6%
0401	12	17%
0402	8	11%
0404	2	3%
0501	8	11%
0502	6	8%
0503	5	7%
0504	1	1%
0505	2	3%

Table 3.21 : HLA DQA1 allelic pool in the Bamileke community

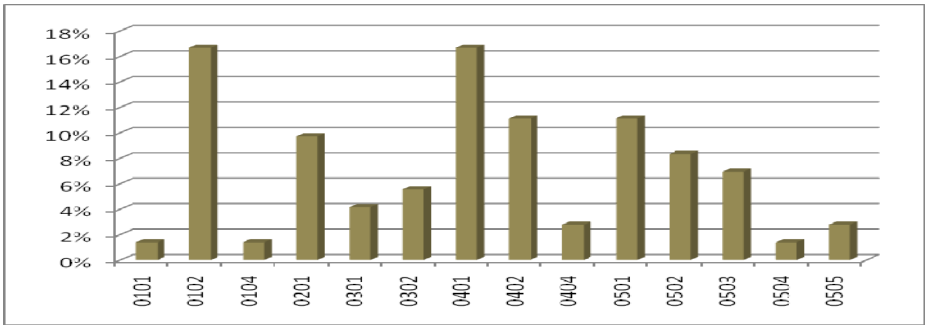


Figure 3.26 : HLA DQA1 allelic distribution in the Bamileke community

The variants at HLA DQB1 reflect the wide variability observed in DQA1 locus: 15 alleles are expressed in Bamileke with frequencies ranging from 1% up to 21% of DQB1*0301 and 17% of *0602 (Table 3.22 and Figure 3.27).

DQB1	*TOTAL*	*%*
0201	9	13%
0202	3	4%
0203	4	6%
0301	15	21%
0302	9	13%
0303	2	3%
0304	1	1%
0317	1	1%
0401	5	7%
0501	7	10%
0502	1	1%
0602	12	17%
0603	1	1%
0616	1	1%
0627	1	1%

Table 3.22 : HLA DQB1 allelic pool in the Bamileke community

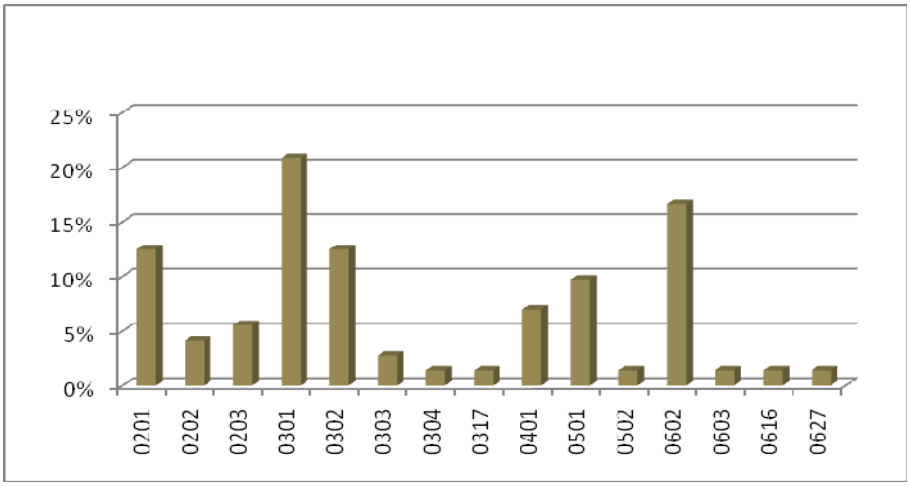


Figure 3.27 : HLA DQB1 allelic distribution in the Bamileke community

As mentioned above, no onchocerciasis information are provided for this population, so it is not possible to correlate each allele, both DQA1 and DQB1, with infectious features or genetic protection. As reported for Tsachilas, this population sample can be supposed to be constitute by apparently healthy people (Scozzari et al., 1994; Spedini et al., 1999).

4. DISCUSSION

4.1 Families

Based on clinical observations and immunological studies, it had been proposed that the variability in disease caused by tissue nematode infections results from qualitative differences in the host's immune response, which depends on invariable individual properties (King et al., 1991). This hypothesis has been commented upon by others who suggested that disease variability and susceptibility may reflect a sequential progression of immune reactions rather than a "static" immunological configuration (Bundy, 1991). By definition, a genetic analysis such as the one presented here could not possibly provide support for the latter model, which is based on changes over time. Nevertheless, the fact that significant associations were obtained between genetic traits and the *O.volvulus* infection supports the former concept of stable phenotypes (Ottesen, 1992). The unique opportunity to analyze two populations of different ethnicity that share a hyper-endemic environment for onchocerciasis may constitute a valuable tool to investigate the role of the immunogenetics in an endemic infection. As mentioned above, the two Esmeraldas populations share the same environment but consist of two quite genetically separated samples, each with a particular immunogenetic background.

The familial samples, in spite of the small sample size, present some interesting focal points. In Afroecuadorian individuals, a link between the HLA DQB1*0301 and a form of protection from infection by *O. volvulus* (Figure 3.11) seems to exist. This result confirms the data already present in the literature (Meyer et al., 1994), which refer only to populations of the African continent. This allele could be subjected to selective pressure. The frequency difference between affected and unaffected individuals is noticeable but not statistically significant, although it may be due to the small sample.

As for the HLA DQA1 locus, the Afroecuadorian community displays the presence of two alleles in individuals supposedly protected from infection (not statistically significant between healthy and onchocerciasis): HLA DQA1*0401 and HLA DQA1*0505 (Table 3.5 and Figure 3.10). These alleles do not match the

hypothetically protective DQA1*0501 allele, found in populations of West Africa, for which there are unique bibliographic data (Meyer et al., 1994 and Donfack et al. 1999). The HLA DQA1*0401 is also present in the healthy portion of the Cayapas families, and this could support the hypothesis of protection provided by this allele ($p = 0.05$). Notwithstanding the sample size, the statistical difference between cases and controls borders on significance. The DQA1 allele *0301 is significantly present in the Cayapas cases, but this association does not appear in the Afroecuadorian families.

The determination of the haplotypes using the software Phase v. 2.1 (Stephens et al., 2001) (Table 4.1) makes it impossible to identify a hypothetical protective association, even if there is a leading frequency of HLA DQA1*0505-HLA DQB1*0301 present in 6 haplotypes among healthy Afroecuadorians. The Cayapas families seem to differ again from Afroecuadorians: in this sample, the HLA DQA1*0401-HLA DQB1*0302 is the leading haplotype among healthy people.

However, in determining a hypothetical state of immunity, one allele rather than a single haplotype seems to be involved, whether belonging to the HLA DQA1 locus or the HLA DQB1 locus. The distribution of HLA DQA1*0401 appears to confirm a peculiar condition. The significantly higher presence of the DQA1*0401 seem to be more involved in conferring protection from infection by *O. volvulus* compared with DQA1*0505, whose hypothetical protective activity could be affected by the action of DQB1*0301.

Haplotype		Afroecuadorians		Indios Cayapa	
HLA DQA1	**HLA DQB1**	**Oncho**	**No Oncho**	**Oncho**	**No Oncho**
*0101	*0302			2	
*0101	*0402				2
*0101	*0316				1
*0101	*0501		1		
*0101	*0611		1		
*0102	*0301		2		
*0102	*0302			1	1
*0102	*0501	1			
*0102	*0502	1	2		
*0102	*0602	3	2		
*0102	*0609	3	2		
*0102	*0611		2		
*0102	*0619		1		
*0201	*0202	1	2		
*0202	*0609		1		
*0301	*0302		2	6	5
*0301	*0303			6	3
*0302	*0302				1
*0302	*0303	2	2	1	5
*0302	*0315				4
*0302	*0402			2	
*0302	*0602	1			
*0303	*0302		1		
*0401	*0301		1	1	
*0401	*0302				6
*0401	*0303		1		2
*0401	*0402	1	3	2	6
*0401	*0316				1
*0401	*0602		2		
*0501	*0302				1
*0501	*0303				2
*0503	*0301		1		4
*0503	*0302		1		
*0503	*0303			3	
*0503	*0316				1
*0505	*0301	1	6	2	1
*0505	*0302		1		
*0505	*0402		1		

Table 4.1 : Estimated haplotypes HLA DQA1-DQB1 in familial samples

This observation is further supported by the distribution of the DQA1*0401 allele in unaffected individuals from both communities at substantial frequencies (18.4% in Afroecuadorians compared to 32.6% in Indians).

By linking the series of results concerning the analysis of polymorphism rs1056315 with the molecular typing of HLA DQB1, the T allele in SNP rs1056315 shows a moderate association with the allele DQB1*0301 (Figure 3.12) in the community of African origin. There is a strong association between the presence of DQB1*0301 and the T allele of SNP rs1056315, but the reverse is not true. This uniqueness in occurrence is confirmed by the low r^2 computed by the software Haploview (Barrett et al., 2005) applied to the familial study. In Afroecuadorians, the r^2 values do not exceed 0.2 (with a LOD=1.07), while the absence of any kind of correlation between the SNP and HLA DQB1*0301 is manifested in Cayapas, with an r^2 of only 0.035 (LOD=0.1). These observations confirm, at least in part, the data reported in the literature (de Bakker et al., 2006) for communities of African descent. In Cayapas, conversely, there does not seem to be an association between the SNP rs1056315 and DQB1*0301. This concept stresses the need for more and more genetic data on molecular polymorphisms in order to significantly contribute to our knowledge of intra- and inter-population genetic variability and to improve the SNP predictiveness regarding HLA variants.

4.2 Population analysis

4.2.1 Cayapas

The population analyses on one side confirm the perceptions provided by the familial study, increasing the number of cases and controls.

Two alleles seem to represent protective variants both in HLA DQA1 and HLA DQB1, suggesting the importance of every locus of HLA DQ in response to onchocerciasis. The protective role of a DQB1 allele is somewhat expected for the importance of β1 chain residues in binding antigen epitopes to the cleft. On the other side, the protective importance of a DQA1 variant stresses the significance of the α1

protein sub-unit to ensure a suitable environment to successfully respond to an Onchocerca attack. As mentioned above, the DQB1*0402 is the protective allele in Cayapas with OR=0.42, but the DQA1*0401 statistically reached OR=0.29, showing a stronger protective effect.

The DQA1 variants also encompass the *0301 allele, for which a susceptibility role may be supposed in accordance with OR=2.31, highlighting again the magnitude of the α1 sub-unit in regulating the immune response to onchocerciasis. The haplotype frequencies were estimated according to Markov chains in Cayapas, and this estimation highlights the leading frequencies of HLA DQA1*0301-DQB1*0302, and HLA DQA1*0401-DQB1*0402 ($p<0.01$) (Figure 4.1).

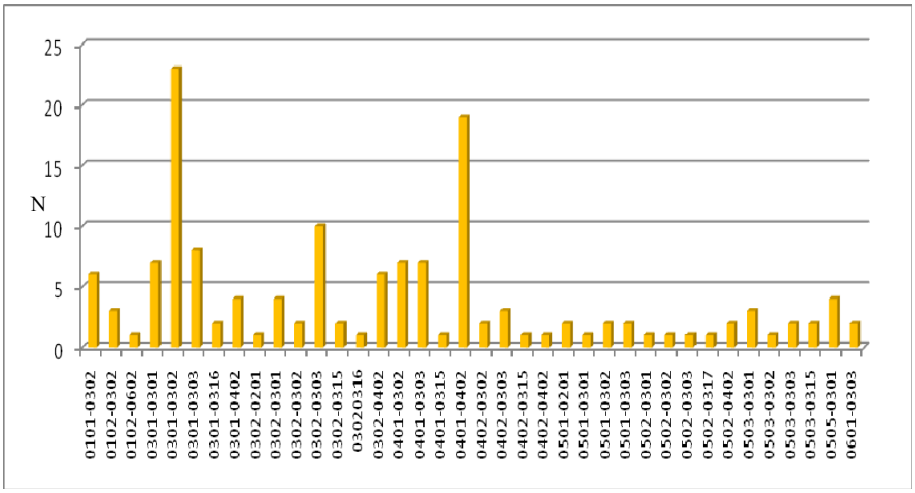


Figure 4.1 : Estimated haplotype counts in Cayapas ($p<0.01$)

The subdivision of the sample according to clinical status shows that haplotype DQA1*0301-DQB1*0302 is present in 17.5% of cases (Figure 4.2), while DQA1*0301-DQB1*0303 reaches the 10% of the total haplotypes. This result underlines the importance of the single allele DQA1*0301 rather than a haplotype in determining a person's susceptibility to onchocerciasis, although the OR is not very strong. In fact the DQA1*0301 DQB1*X alleles represent the 37.5% of the onchocerciasis Cayapas.

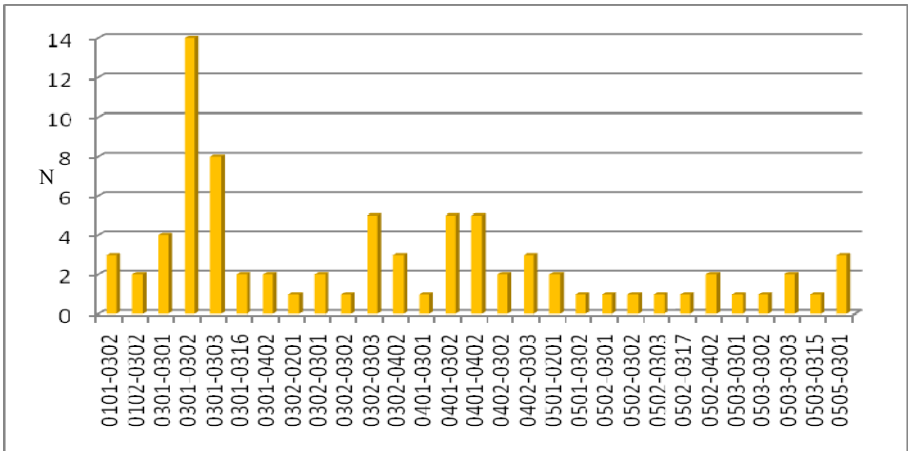


Figure 4.2 : Estimated haplotype counts in onchocerciasis Cayapas ($p<0.01$)

Among the controls, two haplotypes are more often represented (Figure 4.3): DQA1*0401-DQB1*0402 is the most frequent at 20.6%, followed by a surprising DQA1*0301-DQB1*0302 at 14.7%. While the former haplotype is expected because it encompasses both statistically meaningful protective alleles, the latter is one of the best presented in cases, confirming the not so strong susceptibility role of the allele DQA1*0301. This on one side confirm the fundamental protective role of the allele DQA1*0401, of which haplotypes are the 33.8% of the total, and DQB1*0402, of which haplotypes represent the 29.4%,; but on the other side the notable frequency of DQA1*0301-DQB1*0302 suggests a certain dilution effect of DQB1*0302 in the susceptibility effect of HLA DQA1*0301.

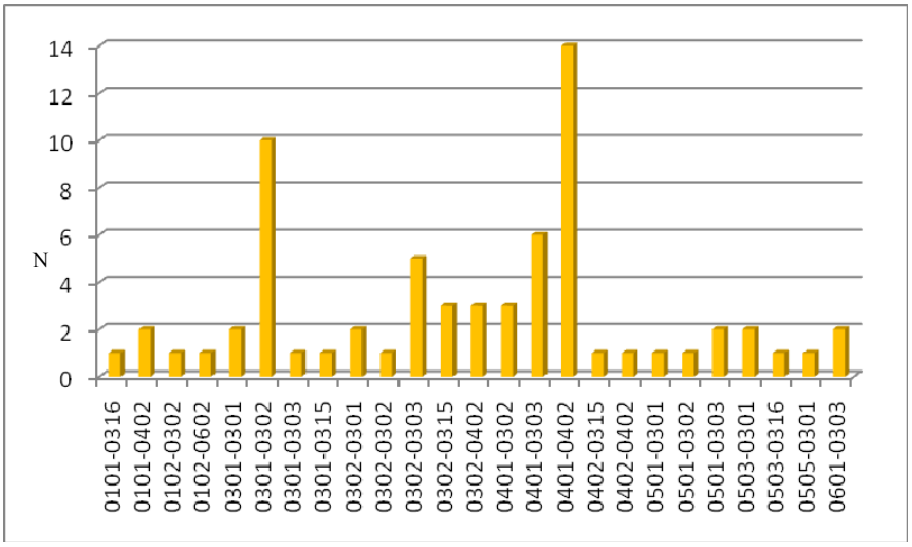


Figure 4.3 : Estimated haplotype counts in healthy Cayapas ($p<0.01$)

4.2.2 Afroecuadorians

The same approach was followed for the Afroecuadorian population sample, in which the hypothesis found based on the familial samples was implemented. In this population sample, the protective role of DQA1*0401 was confirmed by the extreme significance of the allele distribution ($p<0.0001$) between cases and controls, with OR=0.13, which denotes a strong protective role against onchocerciasis.

The DQA1 locus in Afroecuadorians is characterized by the important susceptibility role of two variants encompassed in the *01 class: *0102 and *0103. These alleles are completely differently distributed between Afroecuadorians and Cayapas. In people of African descent, these alleles reach 32% and 11% respectively, while in Cayapas, *0102 accounts for 2% and *0103 is not present. In the Afroecuadorian population, the distribution of such allelels between cases and controls is extremely meaningful for understanding the susceptibility provided by these alleles ($p<0.001$ and $p<0.0001$, respectively). Coversely the Cayapas' susceptibility allele

DQA1*0301 is quite absent in Afroecuadorians, reaching only 1%. Haplotypes were estimated between the DQA1 and DQB1 loci in Afroecuadorians: DQA1*0401-DQB1*0301 is the leading one followed by DQA1*0102-DQB1*0602 (11.3% and 6%, respectively) (Figure 4.4).

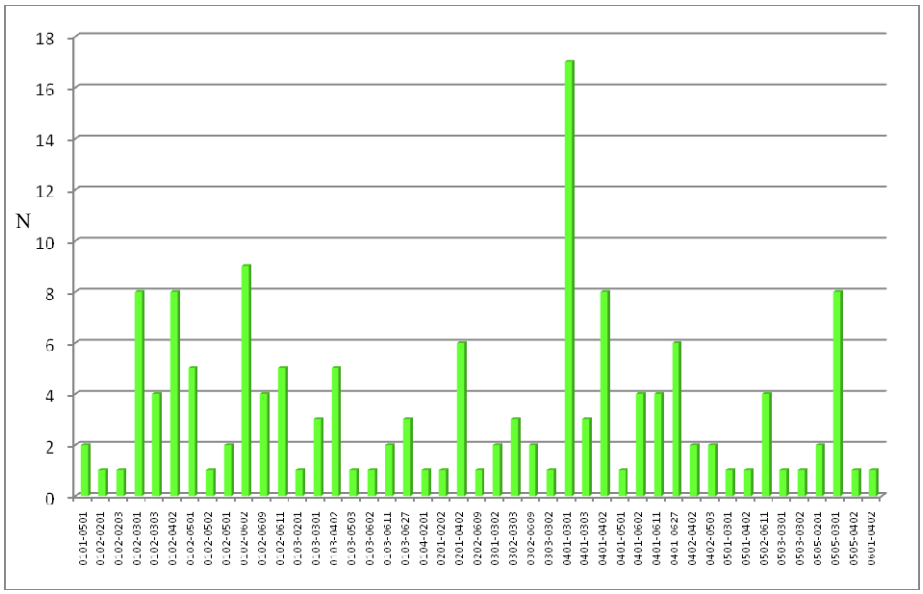


Figure 4.4 : Estimated haplotype counts in Afroecuadorians ($p<0.01$)

All 17 DQA1*0401-DQB1*0301 estimated haplotypes are included in the healthy section of Afroecuadorians (Figure 4.5), it is unsurprising because of the presence of the two major putatively protective alleles in Afroecuadorians. The second haplotype in the controls is DQA1*0401-DQB1*0402, which may suggest a kind of additive role in protection provided by DQB1*0402 even though in Afroecuadorians it does not reach statistical significance in cases/control distributions. Note, however, that the haplotypes that present DQA1*0401 reach 42% of the total haplotypes, highlighting the crucial protective role of this allelic variant versus only 20% of haplotypes sharing the HLA DQB1*0402 allele.

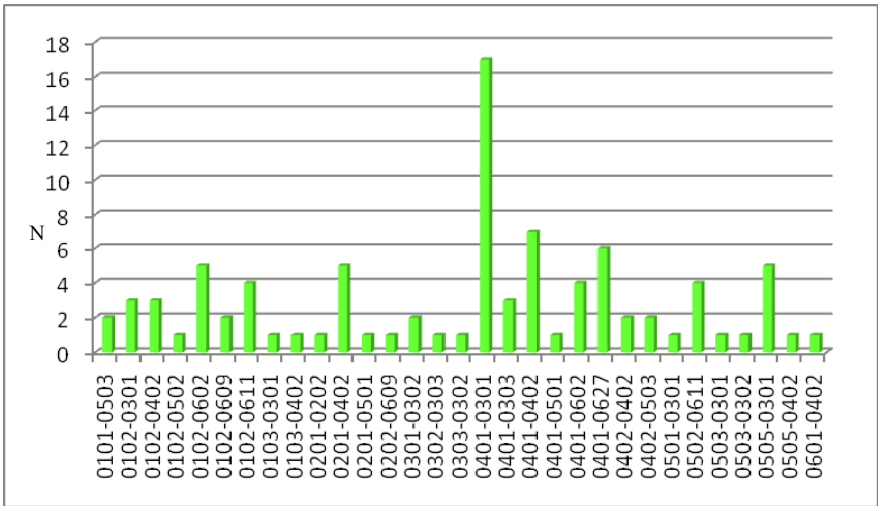


Figure 4.5 : Estimated haplotype counts in healthy Afroecuadorians ($p<0.01$)

Among the affected Afroecuadorians, no one haplotype is predominantly present. The hapotype with the highest frequency is DQA1*0102-DQB1*0301, but it does not exceed 13.3% of the total haplotypes (Figure 4.6). It is therefore clear that the susceptibility role is due to a single allele such as DQA1*0102, whose haplotypes reach 50%, or DQA1*0103, which accounts for a further 23% of the total haplotypes.

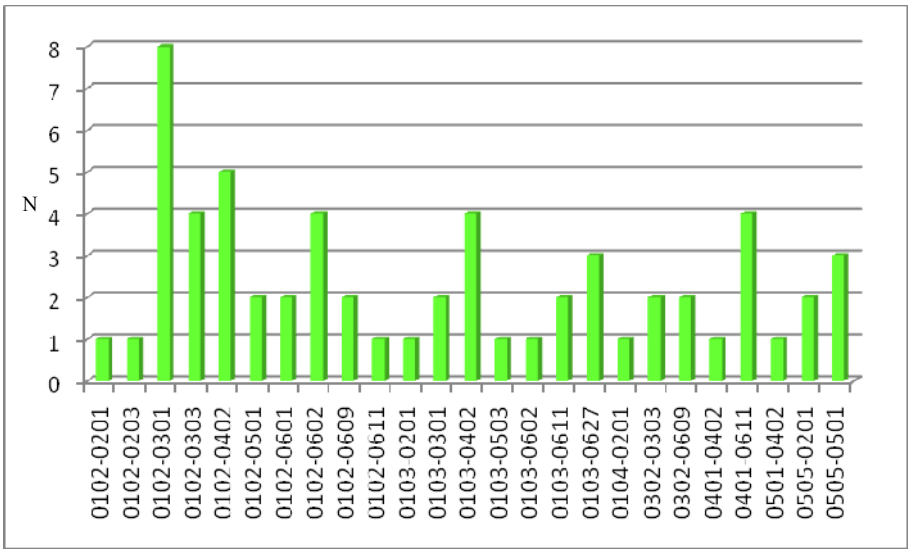


Figure 4.6 : Estimated haplotype counts in onchocerciasis Afroecuadorians ($p<0.01$)

It is also evident in the Afroecuadorians that the HLA DQA1 gene plays more than a secondary role in the immunological response to onchocerciasis.

Thus, if on one side the HLA DQB1 locus encodes for a sub-unit that plays a key role in the antigen epitope binding, the observations in these two separated populations confirm that the chief task of positively/negatively responding to onchocerciasis is reserved for the α1 sub-unit, and the direction of the response, positive or negative, is due to the presence of a particular allelic variant.

As reported above, few HLA-onchocerciasis molecular data are available in the literature, and the only published ones are for African populations like Liberians (Meyer et al., 1994), Nigerians (Murdoch et al., 1997) and Cameroonians (Donfack et al., 1999). In these papers, one can observe a regularity of the findings, and the protective role of a DQA1 allele seems to be constant in the three populations. In every paper, the DQA1*0501 is associated with protection against onchocerciasis. For Liberians, the protection is provided by the haplotype DQA1*0501-DQB1*0301 and there are three susceptibility alleles, all for the DQB1 locus: *0101, *0201 and

*0501. DQA1*0501 is already associated with protection against onchocerciasis in Cameroonians, where the infection is linked to HLA DQB1*0201.

More complexity is showed in Nigerians, where there are several susceptibility alleles in connection with a different form of dermal onchocerciasis: DQB1*0301, *0502 but also DQA1*0101 and *0501. These findings appear to disprove the Liberian and Cameroonian scenario, wherein the haplotype DQA1*0501-DQB1*0301 is the protective one. Indeed, these two alleles are also present in putatively immune individuals identified in these populations: such "immune" individuals are presumed to mount a strong immune response against the infective L3 larval stage and prevent the development of adult worms. The DQA1*0501-DQB1*0301 haplotype therefore appears to be associated with the ability to mount a strong immune response against at least two stages of *O.volvulus* development: the microfilariae (i.e., L1) and L3. It has been proposed that asymptomatic onchocerciasis subjects with high microfilarial loads possess a form of tolerance which, if broken, results in progression to clinical disease (Maizels et al., 1991). In the Esmeraldas samples, these alleles are underrepresented.

The estimation of HLA DQB1-rs1056315 haplotypes highlights the leading presence of the DQB1*0301-rs1056315T (26 haplotypes) (Figure 4.7). This confirm the association preference of the T allele of the SNP regards to HLA DQB1*0301 allele, but this association does not reach sufficient correlation coefficient to be useful to predict the presence of the *0301 allele (r^2=0,2) in Afroecuadorians.

Worse situation is shown in Cayapas, where the HLA DQB1*0301-T allele for rs1056315 is present only in 8 copies (Figure 4.8).. In this sample this association absolutely does not appear and the correlation coefficient is somewhat of very low frequency (r^2=0,026). These observations point out the need to improve data regarding to Tag SNP associated to HLA (de Bakker et al., 2006) in order to predict the HLA variant.

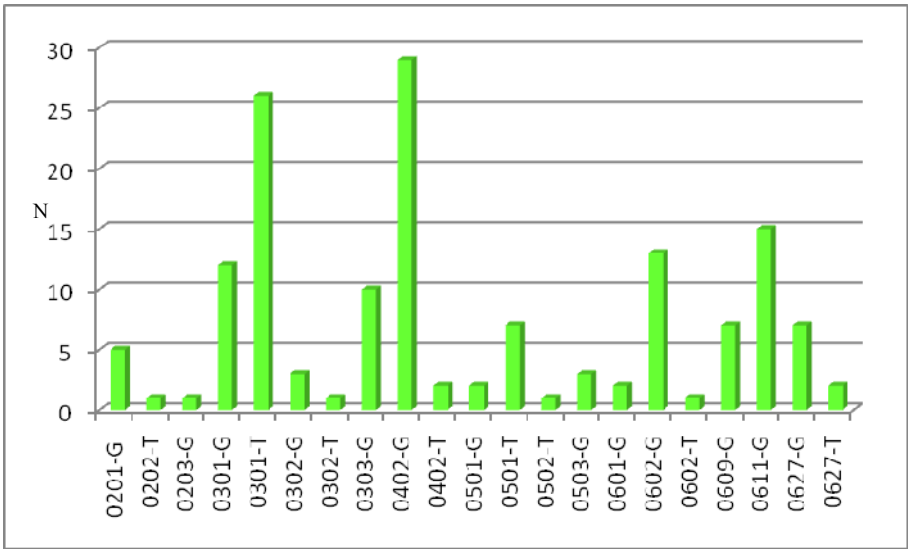


Figure 4.7 : Estimated HLA DQB1-rs1056315 haplotype counts in Afroecuadorians (p<0.01)

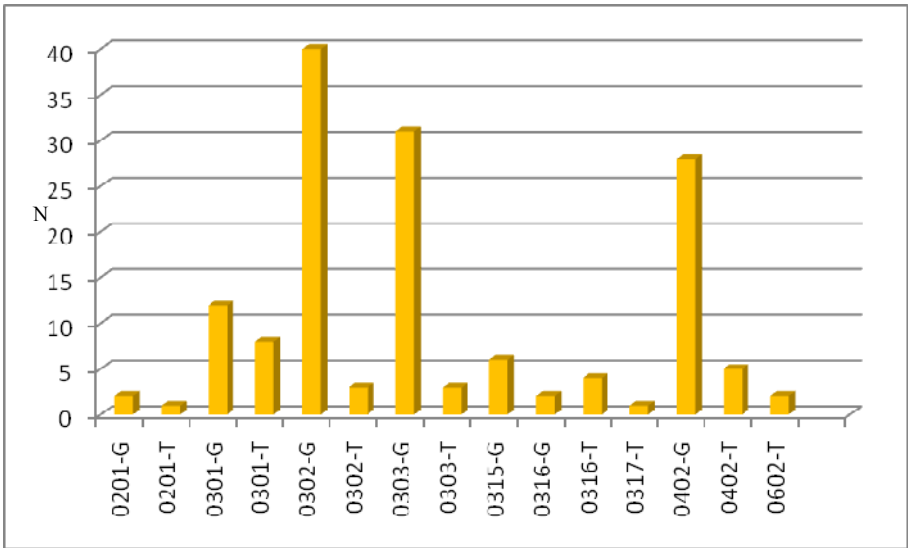


Figure 4.8 : Estimated HLA DQB1-rs1056315 haplotype counts in Cayapas (p<0.01)

4.2.3 Comparative analyses with available populations

In order to graphically display the similarity among populations, a correspondence analysis was carried out comparing the Esmeraldas, Cameroonians (Donfack et al., 1999) and Liberians (Meyer et al., 1994). Nigerians (Murdoch et al., 1997) do not fit the data structure, so they cannot be included in the evaluation.

Correspondence Analysis is a technique that generates graphical representations of the interactions between modalities (or "categories") of two categorical variables, like HLA alleles. It allows the visual discovery and interpretation of these interactions, that is, of the departure from independence of the two variables. Each population was subdivided according to clinical status to allow the identification of some similarities among populations.

The correspondence analysis was conducted by counts of the HLA DQA1 and DQB1 variants in each sample.

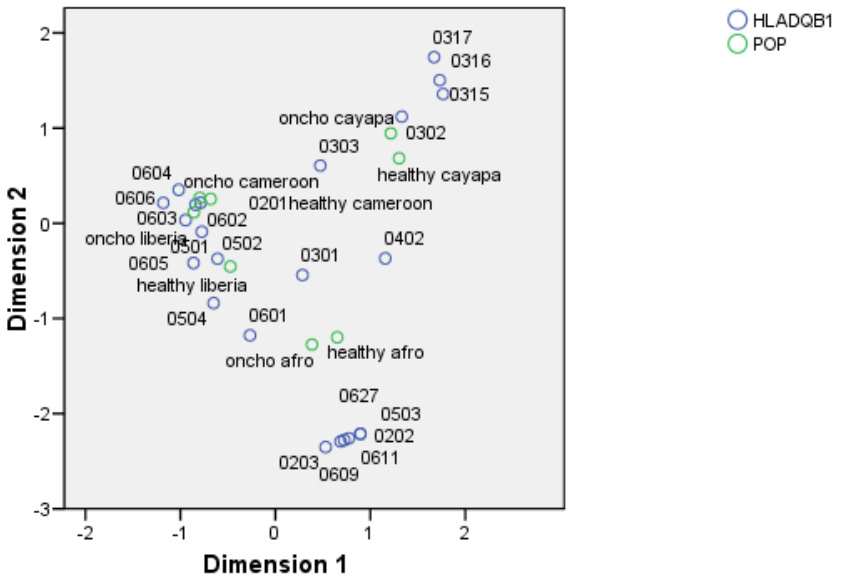


Figure 4.9 : Bidimensional representation of correspondence analysis related to HLA DQB1 alleles

The plots (Figure 4.9) encompass bidimensionally 68% (43.8% and 24.2% respectively in the first and second dimensions) of the total variability expressed at the HLA DQB1 locus. Each population was subdivided according to the clinical onchocerciasis status, and the two infection modes are near each other in every sample, showing the existence of peculiar features in the samples themselves.

Note the correspondence of each allelic variant within the populations, which represent the alleles that may mostly influence the selected group.

The distribution of the samples in the plots ($p<0.001$) shows an aggregate of African populations, near which are located the Afroecuadorians of the Esmeraldas. Distantly placed are the Cayapas, highlighting the different HLA DQB1 composition. HLA DQB1*0301, the hypothetically protective allele in the African samples and, although without statistical significance, also in Afroecuadorians, is placed in the middle of the plot, surrounded by every African or African-descended population. The Cayapas lie further away on the plot, with the healthy group located near *0402, which represents the protective allele in this South American sample. This allele seems to be in an intermediate position between healthy Amerindians and healthy Afroecuadorians, suggesting the influence of this allele in protection in both South American samples.

The same analysis was computed with the HLA DQA1 alleles (Figure 4.10), and in this case the results show an interesting distribution. The plot significantly shows ($p<0.001$) 70.3% of the total variability (47.4% and 22.9%) and again demonstrates the proximity of both clinical groups for each population. The Ecuadorian healthy samples, however, are slightly shifted with respect to the affected ones and are placed near the new hypothetically protective allele *0401, while the susceptibility roles of *0102 and *0103 are confirmed by their proximity on the first dimension between these alleles and the onchocerciasis Afroecuadorians. HLA DQA1*0301 also confirms its susceptibility role in the Cayapas, placing itself near to the onchocerciasis Cayapas.

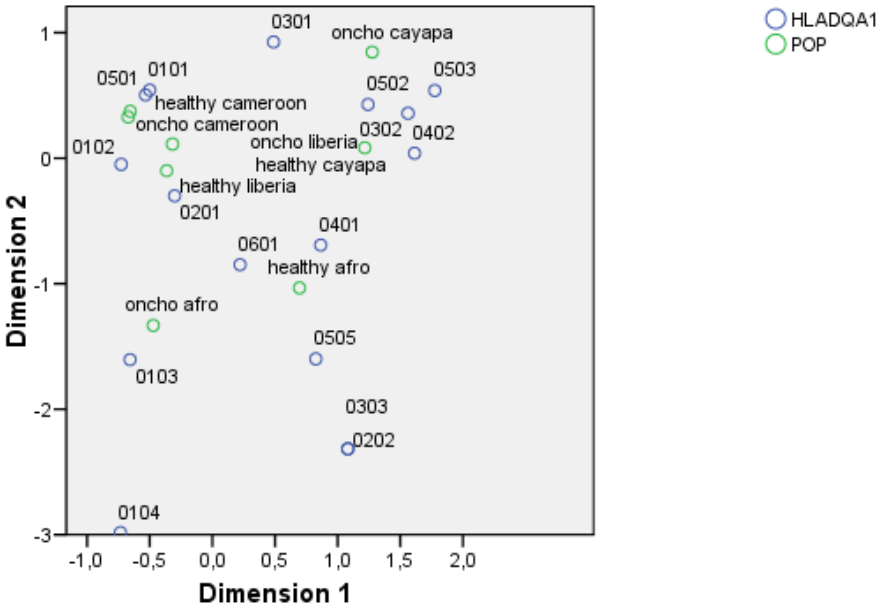


Figure 4.10 : Bidimensional representation of correspondence analysis related to HLA DQB1 alleles

This typing analysis also shows how variable the HLA region is, probably due to a different background. This feature is underlined by the multiallelic structure of the genes, which are maintained by natural selection but also by genetic drift. In the Old World, every population is characterized by numerous alleles with low frequencies; in the Americas, the situation is quite different, because few alleles may reach high frequencies, and others are maintained in somewhat low frequencies (Cavalli-Sforza et al., 1994). Each population may be unique because, as result of genetic drift, the high frequency allele may be different in each population. The high frequencies found for DQA1*0401 in both Esmeraldas samples suggest that this distribution may not be completely due to genetic drift: the separation between populations (De Stefano, 1994; Rickards et al., 1994, Martinez Labarga et al., 1999) shows that migration did not play a significant role in shaping the genetic background of this human group, so the plausible response may be an effective selective pressure that positively selected this DQA1 allele in both populations.

Conversely, the differential findings regarding the susceptibility alleles seem to confirm the above speculation, that the different genetic backgrounds reflect different ethno-geographic origins. It should be noted, however, that the South American scenario is very different than the African one, and it underlines the extreme significance of the environment in modeling the human genetic background.

To compare the immunogenetic arrangement of the Esmeraldas populations, a comparison between them and other populations was carried out: indios Tsachilas, an Amerindian sample, and Bamileke from Cameroon. For these populations, no individual status is provided regarding onchocerciasis, but we can use epidemiological and historical information to characterize these groups. Indios Tsachilas, like the Cayapas, believe themselves to be descended from peoples of the Andean highlands, whereas linguistic evidence seems to indicate an Amazonian origin of the two Ecuadorian populations (Barriga Lopez 1987, Carrasco 1988). Both views support a common ethnogenesis of the Tsachilas and Cayapas although they seem to be quite genetically separated than Cayapas (Babalini et al., 2005). The establishment of a new focus of onchocerciasis was suspected in the Tsachilas because of the in-migration of Cayapas Amerindians over the previous 10 years (1986–1996) and the presence of the vector species *Simulium exiguum* (Charalambous et al., 1997)

The Chachi dispersion has established new potential foci in the Esmeraldas and Pichincha Provinces, where human infection reservoirs and vector species of black fly coexist (Cooper et al., 2001, Guderian et al., 1992). Because this Tsachilas sample was recruited for a population study, they consisted of apparently healthy individuals of both sexes belonging to the communities of Chihuilpe and Congoma.

The Bamileke were similarly recruited as a population sample: only unrelated donors in apparent good health were considered (Spedini et al., 1999). Nevertheless, the Bamileke inhabit the western plateau known also as the ‘‘Bamileke plateau,’’ which, according to the WHO, is classified as an endemic area of onchocerciasis. (WHO, 2006)

Thus the molecular typing of these comparison samples shows how these populations are genetically different.

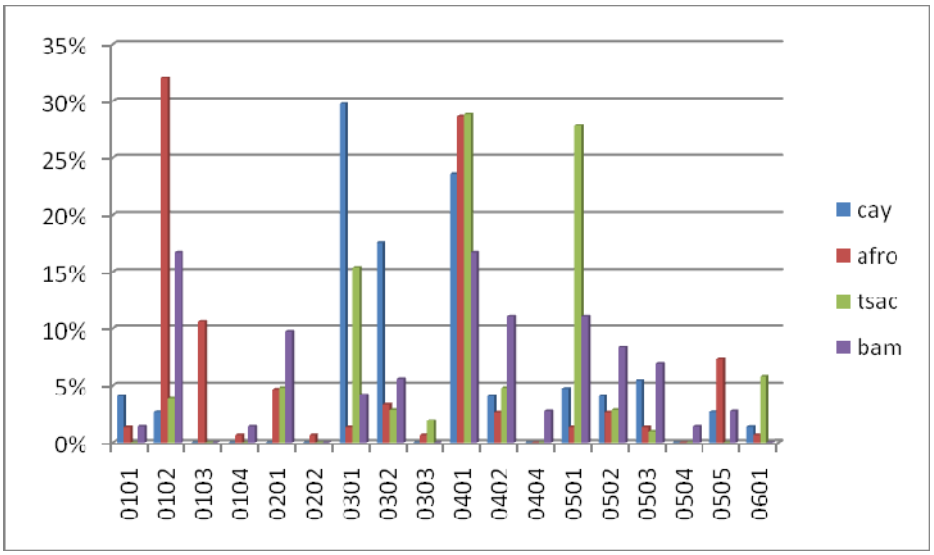


Figure 4.11 : Allele distribution at HLA DQA1 in the four analyzed population

At the HLA DQA1 locus, the differences between the four sample populations appear statistically significant ($p<0.001$) (Figure 4.11). In all the samples, *0401 is present at considerable frequency: while in Tsachilas (29%) it may be due to genetic admixture with Cayapas and positive selection related to onchocerciasis, 17% of the Bamileke may be ascribed to sampling error because the low sample size. Otherwise, the *0501 peak in Tsachilas may confirm Cavalli-Sforza's observation that a single HLA allele in Amerindians could reach notable frequencies (Cavalli-Sforza et al., 1994) through genetic drift. However, a 2004 study (Amirzargar et al., 2004) seems to associate this allele with protection against tuberculosis, a disease not very prevalent in Santo Domingo de los Colorados (Romero-Sandoval et al., 2007).

The same comparison was carried out for the DQB1 locus (Figure 4.12). The allelic distributions are statistically different among populations ($p<0.001$). The leading frequencies of DQB1*03 alleles appear in all sampled populations: in Amerindians

they exceed 50% (Cayapas 69% and Tsachilas 52%), while in Africans and Afroecuadorians they do not reach 40% (Afroecuadorians 36% and Bamileke 37%).

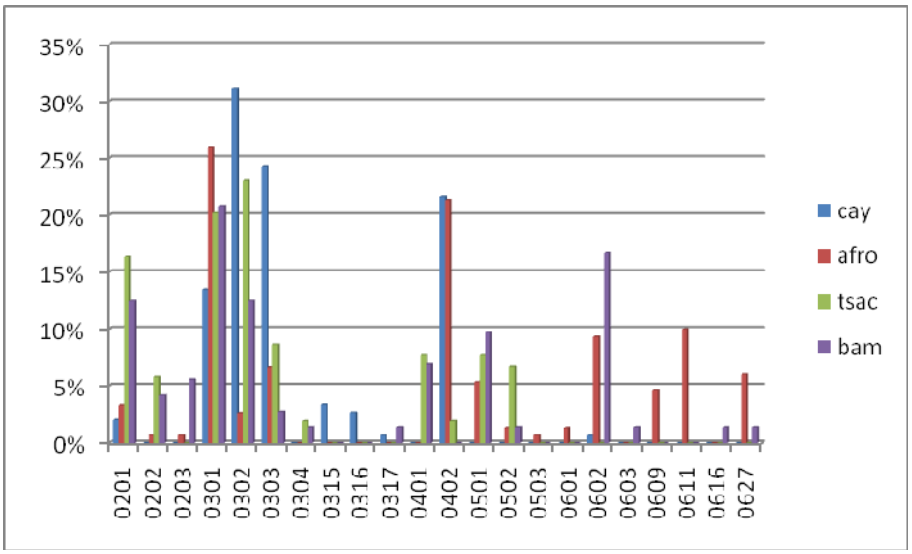


Figure 4.12 : Allele distribution at HLA DQB1 in the four analyzed population

The DQB1*0301 peaks in all populations but the Cayapas may be due to the protective association of this variant with tuberculosis itself (Vejbaesya et al., 2002, Dubaniewicz, 2005). The DQB1*0602 allele in the Bamileke (16%) could be associated with susceptibility to *Mycobacterium* infection in Africa (Zane Lombard et al., 2006), the probable geographical origin of Afroecuadorians of the Esmeraldas Province.

In order to graphically display the differences among populations, correspondence analysis was carried out for both HLA DQA1 and HLA DQB1.

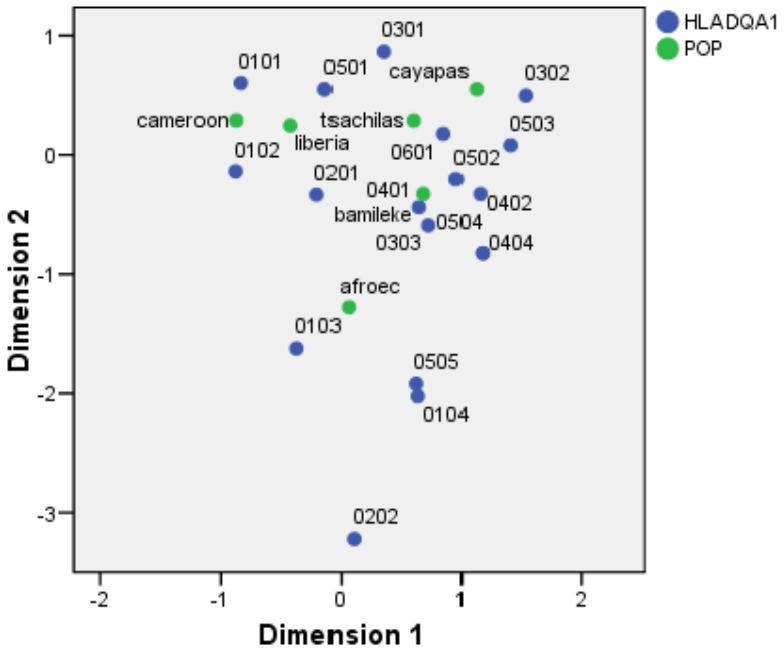


Figure 4.13 : Correspondence analysis of the four DQA1 data sets in the analyzed populations

The HLA DQA1 analysis (Figure 4.13) significantly interprets ($p<0.001$) 68.4% of the total variability by 2D representation (46.8% and 21.6% respectively in the first and second dimensions). The Tsachilas are placed near the Cayapas, while the African samples cluster together with Afroecuadorians, showing how this sample inherits some of the genetic background of the parent African populations. The outlying position of the Afroecuadorians surely is due to the 11% of the *0103 allele.

A different scenario is provided by DQB1 variants (Figure 4.14) (the first two dimensions underlie 37.3% and 22.3%), as the Bamileke, Liberians and Cameroonians cluster in one group and the other population are more shifted, with the Cayapas and Tsachilas sharing a single group different from the Afroecuadorians. This spatial distribution reflects the singularity of the Esmeraldas samples, characterized by the peculiar allele distribution at each HLA DQ locus.

Thus it is clear from all these speculations how much heterogeneity is present in the HLA region. Several polymorphic regions of the human genome have been used in recent years to infer human phylogenies (Cavalli-Sforza 2005).

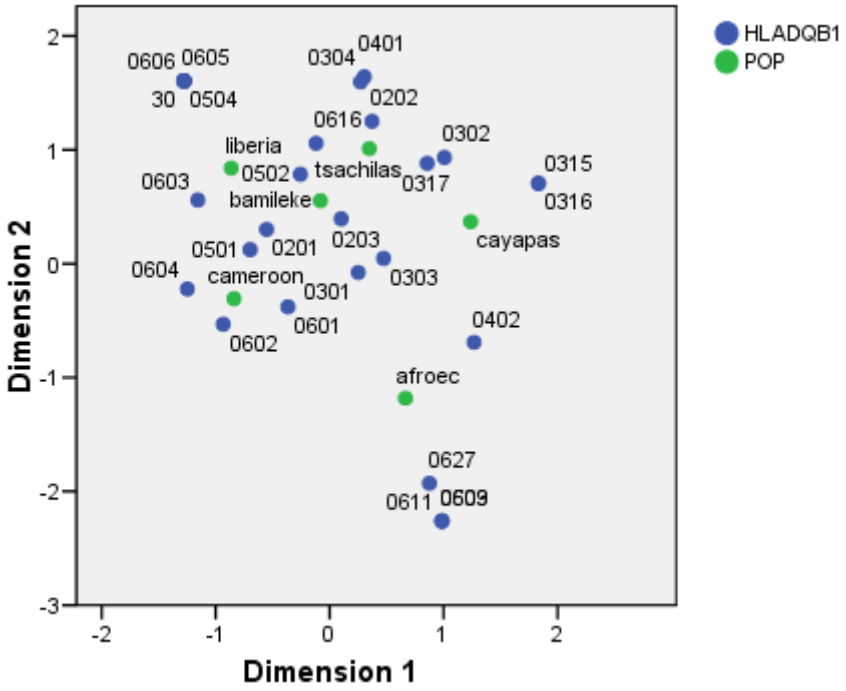


Figure 4.14 : Correspondence analysis of the four DQB1 data sets in the analyzed populations

4.2.4 Attempt of phylogenetic analysis

Like all the regions that exhibit a high degree of polymorphism, HLA could be used for this aim. There are several reasons to believe that HLA class II loci are on par with mt-DNA and Y-chromosome markers in phylogenetic assessment (Bharadwaj et., 2007). They are both vastly polymorphic and highly linked loci that result in

unique linkage disequilibria patterns, signature alleles and haplotype segregation among different populations (Begovitch et al. 1992; Grubic et al. 2000).

So phylogenetic reconstruction is attempted by HLA DQA1 and HLA DQB1 allele frequencies. It bases on maximum likelihood (ML) and the HLA class II loci DQA1 and DQB1 allele frequency distribution using CONTML in PHYLIP v3.5c (Felsenstein 1992). A statistical bootstrap involving 1000 replicates was carried out using SEQBOOT option of PHYLIP v3.5c. Finally, a consensus of 1000 trees was drawn using CONSENSE option of PHYLIP v 3.5.

Esmeraldas samples, without split-up due to clinical status, was compared to Tsachilas (present study, PS), Bamileke (PS), Germans (G Bein, 1997), Amhara (Fort et al., 1998), Oromo (Fort et al., 1998), Indians (Bharadwaj et., 2007), Italians (Lulli et al., 1998) and Chinese sample (Mizuki, 1998).

Based on the allele frequency distribution of the two HLA class II loci, phylogenetic analysis was carried out for 10 and a model based approach (Maximum Likelihood – ML) was used to determine the phylogenetic relationship among different global populations. The ML-phylogenetic tree generated from present data set was drawn by CONTML algorithm that works upon the conjecture that random action of genetic drift is the solitary basis of the differences between allele frequencies in different populations (Felsenstein 1993). An enrooted radial phylogram (Maximum Likelihood) is shown in Figure 4.15.

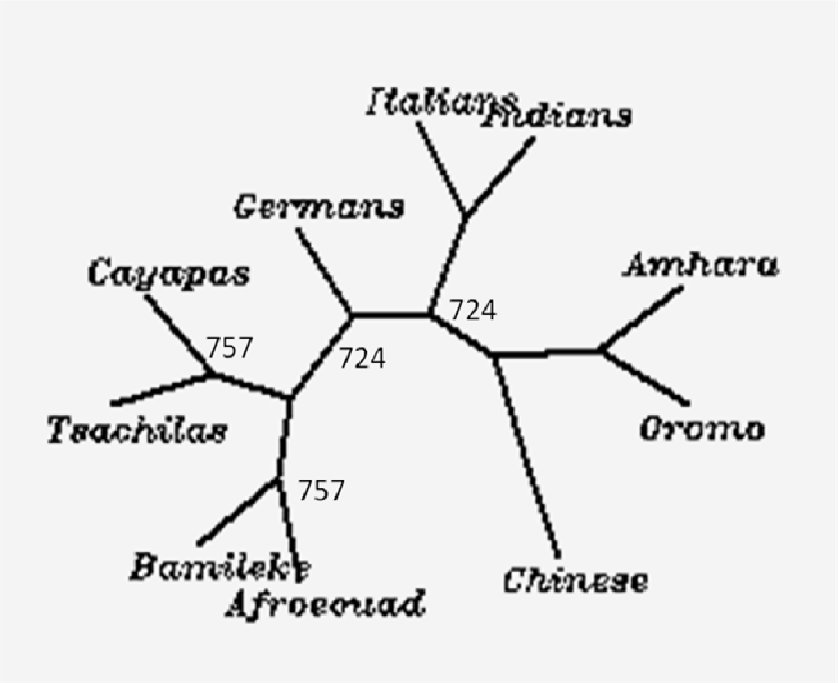


Figure 4.15 : ML phylogram based on HLA DQA1-HLA DQB1 allele frequencies. The numbers refer to occurrence in 1000 replicates of the analysis.

The scores close to the nodes represent the number of bootstrap replicates (out of 1000) exhibiting these specific bifurcations.

The populations spread themselves almost according to bio-geographical criteria: Amerindians cluster themselves while Afroecuadorians share the group with african Bamileke. This distribution may reflect the possible relatedness between people from Guinea gulf and the Africans who reached American coasts during the XVII century slave trade. The genetic background of the new immigrants, since this time, was modified by genetic drift but also by the New World environment that moves from African background. This processes are responsible behind the genetic differences between African and non- African populations and has typically shaped the present day genetic variation (Cavalli-Sforza et a., 1994; Cavalli-Sforza and Feldman 2003). Moreover Europeans cluster themselves with Northern Indians, as

previously reported by literature (Bharadwaj et., 2007). Surprising is the Ethiopian samples and Chinese position, for which an epidemiological hypothesis may be formulated: in fact in both region, Ethiopia and China, malaria foci are present, so that selective stress factor may play an important role in modeling the HLA class II genetic background.

This first attempt to employ ML (Maximum Likelihood) does not reach the surprising results provided by Bharadwaj et collegues (Bharadwaj et., 2007) but it may open a new evaluation process to analyze human similarity. Of course the analysis of the two HLA class II loci depicted a not so strong geo-ethnic phylogeny but indicates that the presence of numerous alleles (against bi-allelic SNPs of mt-DNA and Y-chromosome) does not totally interfere with phylogenetic information content of the loci, provided that frequency distribution of the populations is significantly different. Instead it increases the chance of presence of signature alleles and specific haplotypes in case of closely linked loci like HLA. However, there are potential problems associated with the use of HLA loci in inferring human phylogenies. The most important of them, in general, is the non-uniformity of HLA genotyping methodologies as some studies are based on low resolution typing while others on high resolution. Another discrepancy is in the choice of loci as only few studies involve analysis of all HLA class I and class II loci. Preferably, if all the HLA loci (both class I and class II, if not, at least all class II loci) are analyzed with high resolution of all the alleles and proper statistical interpretation based on more logistic approaches is carried out, then HLA loci can be a suitable marker to infer genetic differences between inter and intra geo-ethnic groups.

5. CONCLUDING REMARKS

It has been more than 30 years since the first report that susceptibility to a disease was associated with inheritance of a specific HLA (Human Leukocyte Antigen) gene.

The association between HLA alleles and a disease may be quantified by typing the HLA alleles expressed by individuals with the disease and the HLA alleles of the general population. Linkage and association studies are the two major types of investigations to determine the contribution of genes to disease susceptibility (or any other phenotype).

The ultimate goal of HLA association studies is to find out how genes cause the disease or modify susceptibility or course of it. One of the many reasons for undertaking gene association studies is to identify disease-specific susceptibility (risk) and protective markers that can be used in immunogenetic profiling, risk assessment and therapeutic decisions.

Resistance to infectious diseases is likely to involve a complex array of immune response and other genes with variants that impose subtle but significant consequences on gene expression or protein function. HLA associations with infectious diseases have been difficult to identify, may be because of complex array of antigenic epitopes is involved in infectious disease pathogenesis. The extensive polymorphism at the HLA loci is thought to have arisen through natural selection by infectious diseases, operating on the diversity generated by mutation, gene conversion and recombination.

In Esmeraldas focus of onchocerciasis the two quite completely separated populations of Cayapas and Afroecuadorians share the same environment but display a different clinical manifestation.

Different allelic composition at HLA DQA1 and DQB1 loci in the two populations are highlighted: of course this most likely seems due to their different ethnic belonging and could be responsible for the observed different clinical display of onchocerciasis.

In Afroecuadorian sample two susceptibility alleles seem to be responsible of the disease, both of DQA1 locus,*0102 and *0103: they are statistically differently distributed between cases and controls.

Affected Cayapas, otherwise, share a different susceptibility allele, the DQA1*0301.

Conversely in both population there are strong evidences related to the protective role of another DQA1 allele: the DQA1*0401, in fact, in both population seems to be significantly distributed in healthy people.

Along this allele, another variants may improve the role of *0401 in Cayapas, the HLA DQB1*0402. This is the unique DQB1 allele statistically distributed among cases and controls in both population.

These findings represent a new positive/negative association with onchocerciasis, in respect to reference data, pertaining only African samples.

The lack of an all-inclusive correlation may be due to superimposed variables such as the kinetics of infective doses or the dose of infection itself. Our data cannot provide any mechanistic explanations for the immunological findings characteristic for the various forms of *O. volvulus* infection. However these data may give rise to some speculations about the key role of HLA DQA1 locus in response to nematode infection.

The association between DQB1*0301 and a tag SNP identified in references, rs1056315, do not represent a strong confirmation to this predictive purpose: in fact in Cayapas the correlation DQB1*0301-rs1056315T does not appear. In Afroecuadorians the correlation seems to be more strong, but the uniqueness of the correlation with T allele does not allow to exceed the r^2 more than 0.2, too bad for representing a predictive tool.

The comparison analyses highlight how the Ecuadorian population differ from the African ones: Cayapas and Afroecuadorians share common features probably linked to the same environment, that of course played a significant role in modeling the different genetic background of these population.

The phylogenetic attempt, although not comprehensive, may be useful to develop a new aspect of the HLA studies, but the uniformity of the typing results and the total HLA loci characterization is a necessary requirement for this kind of analysis.

6. REFERENCES

Abbas, A.K. Lichtman, A.H., and Pober, J.S. (2002). Immunologia cellulare e molecolare. 4th Italian ed. (Piccin), pp. 69-70.

Alexander, N.D., Cousens, S.N., Yahaya, H., Abiose, A., Jones, B.R. (1983). Ivermectin dose assessment without weighing scales. Bull World Health Organ.71:361-6.

Altshuler, D., Brooks, L.D., Chakravarti, A., Collins, F.S., Daly, M.J., Donnelly, P. (2005). A haplotype map of the human genome. Nature 437, 1299–1320.

Amirzargar, A.A., Yalda, A., Hajabolbaghi, M., Khosravi, F., Jabbari, H., Rezaei, N., Niknam,M.H., Ansari, B., Moradi, B., Nikbin, B.(2004) The association of HLA-DRB, DQA1, DQB1 alleles and haplotype frequency in Iranian patients with pulmonary tuberculosis. Int J Tuberc Lung Dis. 8:1017-21.

Awadzi, K. (1980). The chemiotherapy of onchocerciasis II. Quantitation of the clinical reaction to microfilaricides. Ann. Trop. Med. Parasitol. 7, 189-197.

Awadzi, K., Dadzie, K.Y., Shulz-Key, H., Haddock, D.R., Gilles, H.M., Aziz, M.A. (1985) The chemotherapy of onchocerciasis X. An assessment of four single dose treatment regimes of MK-933

(ivermectin) in human onchocerciasis. Ann Trop Med Parasitol. 79:63-78.

Awadzi, K. (2003). Clinical picture and outcome of Seriuos Adverse Events in the treatment of onchocerciasis. Filaria J. 2, 6.

Babalini, C., Tarsi, T, Martinez-Labarg, C.,Scano, G., Pepe, G., De Stefano, G.F., Rickards, O. (2005). COL1A2 (type I collagen) polymorphisms in the Colorado Indians of Ecuador. Ann. Hum. Biol. 32: 666–678.

Bain, C., Merrouche, Y., Puisieux, I., Blay, J.Y., Negrier, S., Bonadona, V., Lasset, C., Lanier, F., Duc, A., Gebuhrer, L., Philip, T., Favrot, M.C. (1997). Correlation between clinical response to interleukin 2 and HLA phenotypes in patients with metastatic renal cell carcinoma. Br J Cancer.75:283-6.

Baker, P.T. (1988). Human adaptability. In: Harrison, G.A., Tanner, J.M.,Pilbeam DR, Baker PT, editors. Human biology: An introduction to human evolution, variation, growth, and adaptability. (Oxford: Oxford University Press), pp. 437–447.

Bharadwaj, U., Khan,F., Srivastava S., Goel, H., Agrawal, S. (2007). Phylogenetic Applications of HLA Class II Loci. Int J Hum Genet, 7: 123-131.

Barriga Lopez F. (1987). Cayapas o Chachis, Vol 4: Etnologia ecuatoriana. Quito: Graficas Duque. Instituto Ecuatoriano de Credito Educativo y Becas

Barrett, J.C., Fry, B., Maller, J., Daly, M.J. (2005). Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics.21:263-5.

Begovich, A.B., McClure, G.R., Suraj, V.C., Helmuth, R.C., Fildes, N., Bugawan, T.L., Erlich, H.A., Klitz, W. (1992). Polymorphism, recombination, and linkare disequilibrium within the HLA class II region. J Immunol, 148: 249-258.

Benzecri, J.P. (1992) Correspondence Analysis Handbook, New York: Marcel Dekker

Blacklock, D. B., (1926). The further development of Onchocerca volvulus in Simulium damnosum. Ann. Trop. Med. Parasitol., 20: 203-218.

Blanks, J., Richards, F., Beltrán, F., Collins, R., Alvarez, E., Zea Flores, G., Bauler, B., Cedillos, R., Heisler, M., Brandling-Bennett, D., Baldwin, W., Bayona, M., Klein, R., Jacox, M. (1998). The Onchocerciasis Elimination Program for the Americas: a history of partnership. Rev Panam Salud Publica. 3:367-74.

Botto, C., Gillespie, A.J., Vivas-Martinez, S., Martines, N., Planchart, S., Masanez, M.G., and Bradley, J.E. (1999). Onchocerciasis hyperendemic in the Unturan Mountains: the value of recombinant antigens in describing a new transmission area in southern Venezuela. Trans. R. Soc. Trop. Med. Hyg. 93, 25-30.

Braasch, D.A., Corey, D.R. (2001). Locked nucleic acid (LNA): fine tuning the recognition of DNA and RNA. Chem Biol. 8:1-7.

Brieger, W.R., Awedoba, A.K., Eneanya, C.I., Hagan, M., Ogbuagu, K.F., Okello, D.O., Ososanya, O.O., Ovuga, E.B., Noma, M., Kale, O.O., Burnham, G.M., Remme, J.H. (1998). The effects of ivermectin on onchocercal skin disease and severe itching: results of a multicentre trial. Trop Med Int Health. 3:951-61

Brown, K.R., Neu, D.C. (1990). Ivermectin--clinical trials and treatment schedules in onchocerciasis. Acta Leiden. 59:169-75..

Bryceson, A.D.M. (1976). What happens when microfilaria die? Trans. R. Soc. Trop. Med. Hyg. 85, 397-399.

Budowle, B., Smith, J., Moretti, T., and Di Zinno, J.(2000). DNA typing protocols: Molecular Biology and Forensic analysis. (Techiques® Books Publication, Eaton Publishing).

Bundy, D. A. P., Grenfell, B. T., Rajagopalan, P. K. (1991). Immunol. Today 12, A71-A75.

Carrasco E. (1988). El pueblo Chachi. El jeengume avanza. Quito: Ediciones Abya-Yala.

Cavalli-Sforza, L.L., Menozzi, P., and Piazza, A.(1994). The History and Geography of Human Genes. (Princeton University Press).

Cavalli-Sforza, L.L., Feldman, M.W. (2003). The application of molecular genetic approaches to the study of human evolution. Nature Genetics 33, 266 – 275.

Cavalli-Sforza, L.L. (2005). The human genome diversity project: past, present and future. Nat Rev Genet, 6: 333-40.

Chan, C.C., Nussenblatt, R.B., Kim, M.K., Palestine, A.G., Awadzi, K., and Ottesen, E.A. (1987). Immunopathology of ocular onchocerciasis. Ophthalmology 94, 439-443.

Charalambous, M., Shelley, A.J., Arzube, M. (1997). The potential for dispersal of onchocerciasis in Ecuador in relation to the distribution of the vector Simulium exiguum (Diptera:Simuliidae). Mem Inst Oswaldo Cruz. 92:153–6.

Collins, R.C., Gonzales-Peralta, C., Castro, J., Zea-Flores, G., Cupp, M.S., Richards, F.O. Jr, Cupp, E.W. (1992). Ivermectin: reduction in prevalence and infection intensity of Onchocerca volvulus following biannual treatments in five Guatemalan communities. Am J Trop Med Hyg. 47:156-69.

Cooper, P.J., Mancero, T., Espinel, M., Sandol, C., Lovato, R., Guderian, R.H., and Nutman T.B. (2001). Early human infection with Onchocerca volvulus is associated with an enhanced parasite-specific cellular immune response. J. Infect. Dis. 183, 1662-1668.

Cordovado, S.K., Hancock, L.N., Simone, A.E., Hendrix, M., Mueller, P.W. (2005). High-resolution genotyping of HLA-DQA1 in the GoKinD study and identification of novel alleles HLA-DQA1*040102, HLA-DQA1*0402 and HLA-DQA1*0404. Tissue Antigens. 65: 448–458.

de Bakker, P.I., McVean, G., Sabeti, P.C., Miretti, M.M., Green, T., Marchini, J., Ke, X., Monsuur, A.J., Whittaker, P., Delgado, M., Morrison, J., Richardson, A., Walsh, E.C., Gao, X., Galver, L., Hart, J., Hafler, D.A., Pericak-Vance, M., Todd, J.A., Daly, M.J., Trowsdale, J., Wijmenga, C., Vyse, T.J., Beck, S., Murray, S.S., Carrington, M., Gregory, S., Deloukas, P., and Rioux, J.D. (2006). A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. Nat. Genet. 38, 1166-72.

De Sole, G., Remme, J., Awadzi, K., Accorsi, S., Alley, E.S., Ba, O., Dadzie, K.Y., Giese, J., Karam, M., Keita, F.M. (1989). Adverse reactions after large-scale treatment of onchocerciasis with ivermectin: combined results from eight community trials. Bull World Health Organ. 67:707-19.

De Stefano, G.F. (1994). Biodiversity and selection in man: an example of biodiversity maintenance by non- adaptive factors. Biol. Internation. Spec. Issue, 32, 63-71.

Donfack, J., Ngu, J.L., Lando, G., Zimmerman, P.A., Nutman, J., Same-Ekobo, A. (1999). Variations under genetic control of onchocerca infection as a function of clinical profile in the endemic center of Cameroon. Bull Soc Pathol Exot. 92:85-90.

Dubaniewicz, A., Lewko, B., Moszkowska, G., Zamorska, B., Stepinski, J. (2000). Molecular subtypes of the HLA-DR antigens in pulmonary tuberculosis. Int J Infect Dis.4:129-33.

Felsenstein J.(1992). Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. Genet Res. 60:209-20.

Fort, M., De Stefano, G.F., Cambon-Thomsen, A., Gira Ido-Alvarez, P., Dugoujon, J.M., Ohayon E., Scano, G., Abbal, M. (1998). HLA

class II allele and haplotype frequencies in Ethiopian Amhara and Oromo populations. Tissue Antigens 51: 327-336.

Garrod, A. (1989). Inborn Factors in Disease, C. R. Scriver and B. Childs eds. (Oxford Univ. Press, Oxford)

Greene, B.M., Taylor, H.R., Cupp, E.W., Murphy, R.P., White, A.T., Aziz, M.A., Schulz-Key, H., D'Anna, S.A., Newland, H.S., Goldschmidt, L.P. (1985). Comparison of ivermectin and diethylcarbamazine in the treatment of onchocerciasis. N Engl J Med. 18:133-8.

Grubic, Z., Zunec, R., Cecuk-Jelicic, E., Kerhin-Brkljacic, V., Kastelan, A. (2000). Polymorphism of HLA-A, -B, -DRB1, -DQA1 and -DQB1 haplotypes in a Croatian population. Eur J Immunogenet, 27: 47-53.

Guderian, R.H., Molea, J., Swanson, D., Proano, S.R., Carrello, D.R., and Swanson, W.L. (1983). Onchocerciasis in Ecuador. I. Prevalence and distribution in the province of Esmeraldas. Tromped. Parasit. 34, 143-148.

Guderian, R.H., Swanson, D., Carrello, D.R., Proano, S.R., Molea, J., and Swanson, W.L. (1983). Onchocerciasis in Ecuador. II. Epidemiology of the endemic foci in the province of Esmeraldas. Tromped. Parasit. 34, 149-154.

Guderian, R.H., Beck, J.B., Stone, D.J., Isabel, K., and Mackenzie, C.D. (1988). Onchocerciasis in Ecuador: recent observations in the province of Esmeraldas. J. Trop. Med. Hyg. 91, 161-168.

Guderian, R.H., Beck, J.B., Proano, S.R., and Mackenzie, C.D. (1989). Onchocerciasis in Ecuador, 1980-1986: epidemiological evaluation of the disease in the Esmeraldas Province. Eur. J. Epidemiol., 294-302.

Guderian, R.H., Shelley, A.J. (1992). Onchocerciasis in Ecuador: the situation in 1989. Mem Inst Oswaldo Cruz. 87:405–15.

Habbema, J.D., Jozefzoon, E., Van Oortmarssen G.J. (1992). Towards the use of decision sciences in leprosy control. Lepr Rev. 63 Suppl 48-52.

Hagan, P., Chauhan, V., Chitnis, C. (1998).Mosquitoes and Monsoon Parasitol Today. 14:169-172.

Haldane,J. B. S. (1948) The theory of a cline. J. Genet. 48: 277-284

Hall, L.R., Pearlman, E. (1999). Pathogenesis of onchocercal keratitis (River blindness). Clin Microbiol Rev. 12:445-53.

Harris, E.E., and Meyer, D. (2006). The molecular signature of selection underlying human adaptations. Am. J. Phys. Anthropol. 43, 89-130.

Harrison, G.A. (1988). Human adaptations. In Harrison, G.A., Tanner, J.M., Pilbeam, D.R., Baker, P.T., editors. Human biology: An introduction to human evolution, variation, growth, and adaptability. (Oxford: Oxford University Press). pp. 145–336.

Hill, A.V. (1996). Genetics of infectious disease resistance. Curr Opin Genet Dev. Jun;6(3):348-53.

Hinds, D.A., Stuve, L.L., Nilsen, G.B., Halperin, E., Eskin, E., Ballinger, D.G., Frazer, K.A., Cox, D.R. (2005). Whole-genome patterns of common DNA variation in three human populations. Science 307, 1072–1079.

Hirayama, K., Matsushita, S., Kikuchi, I., Iuchi, M., Ohta, N., and Sasazuki, T. (1987). HLA-DQ is epistatic to HLA-DR in controlling the immune response to schistosomal antigen in humans. Nature 327, 426-30.

Jobling, M.A., Hurles, M.E., Tyler-Smith, C. (2004) Human Evolutionary Genetics: Origins, Peoples and Disease. New York: Garland Science, 2004.

King, C. L. & Nutman, T. B. (1991) in Immunoparasitology Today, eds. Ash, C. & Gallagher, R. B. (Elsevier, Cambridge, U.K.), pp. A54-A58.

Kennedy, M.W., Fraser, E.M., and Christie, J.F. (1991). MHC class II (I-A) region control of the IgE antibody repertoire to the ABA-1 allergen of the nematode Ascaris. Immunology 72, 577–579.

Kluxen, G., Hoerauf, A. (2008) The significance of some observations on African ocular onchocerciasis described by Jean Hissette (1888-1965). Bull Soc Belge Ophtalmol. 307:53-8.

Kumar, R., Singh, S.K., Koshkin, A.A., Rajwanshi, V.K., Meldgaard, M., Wengel, J. (1998). The first analogues of LNA (locked nucleic acids): phosphorothioate-LNA and 2'-thio-LNA. Bioorg Med Chem Lett. 18:2219-22.

Lewalen, S., Courtright, P. Blindness in Africa: present situation and future needs.(2001) Br J Ophthalmol. 85,897-903..

Lombard, Z., Dalton, D.L., Venter, P.A., Williams, R.C., Bornman, L. (2006). Association of HLA-DR, -DQ, and vitamin D receptor alleles and haplotypes with tuberculosis in the Venda of South Africa. Hum Immunol. 67:643-54.

Lulli, P., Grammatico, P., Brioli, G., Catricala, C., Morellini, M., Roccella, M., Mariani, B., Pennesi, G., Roccella, F., Cappellacci, S., Trabace, S. (1998). HLA-DR and –DQ alleles in Italian patients with melanoma. Tissue Antigens 51: 276-80.

Mackenzie, C.D., Williams, J., Guderian, R.H., and O'Day, J. (1987). Clinical responses in human onchocerciasis: parasitological and immunological implications. Ciba Found Symp. 127,
46-72.

Malkki, M., Single, R., Carrington, M., Thomson, and G., Petersdorf, E. (2005). MHC microsatellite diversity and linkage disequilibrium among common HLA-A, HLA-B, DRB1 haplotypes: implications for unrelated donor hematopoietic transplantation and disease association studies. Tissue Antigens 66, 114-24.

Martin, M., Carrington, M., and Mann, D. (1992). A method for using serum or plasma as a source of DNA for HLA typing. Hum. Immunol. 33, 108-13.

Martinez Labarga, C., Rickards, O., Scacchi, R., Corbo, R. M., Biondi, G., Pena, J.A., Varas De Viera, C., Guevara, A.E.B., Mesa Santurino, M.S., and De Stefano, G.F. (1999). Genetic population structure of two african-ecuadorian communities of Esmeraldas. Am. J. Phys. Anthropol. 109,159-174.

Meyer, C.G., Gallin, M., Erttmann, K.D., Brattig, N., Schnitrger, L., Gelhaus, A., Tannich, E., Begovich, A.B., Erlich, H.A., and Horstmann R.D. (1994). HLA-D alleles associated with generalized disease, localized disease, and putative immunity in Onchocerca volvulus infection. Proc. Nati. Acad. Sci. USA. 91, 7515-7519.

Miller, S.A., Dykes, D.D., and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from nucleated cells. Nucleic Acids Res. 16, 1215.

Miretti, M.M., Walsh, E.C., Ke, X., Delgado, M., Griffiths, M., Hunt, S., Morrison, J., Whittaker, P., Lander, E.S., Cardon, L.R., Bentley, D.R., Rioux, J.D., Beck, S., and Deloukas, P. (2005). A high-resolution linkage-disequilibrium map of the human major histocompatibility complex and first generation of tag single-nucleotide polymorphisms. Am. J. Hum. Genet. 76, 634-46.

Mizuki, N., Ohno, S., Ando, H., Sato, T., Imanishi, T., Gojobori, T., Ishihara, M., Goto, K., Ota, M., Geng, Z., Geng, L., Li, G., Inoko, H. (1998). Major histocompatibility complex class II alleles in an Uygur population in the Silk Route of Northwest China. Tissue Antigens 51:287-92.

Mullis, K.B., Faloona, F., Scharf, S., Saiki, R.K., Horn, G., and Erich, H. (1987). Specific Enzymatic Amplification of DNA in vitro: the

polymerase chain reaction. In Cold Spring Harb. Symp.Quant. Biol. 51, pp. 263-273.

Murdoch, M. E. (1992). The skin and the immune response in onchocerciasis. Trop. Doct. 22, 44-55.

Murdoch, M.E., Hay, R.J., Mackenzie, C.D., Williams, J.M F., Ghalib, H.W., Cousens, S., Abiose, A., and Jones, B.R. (1993). A clinical classification and gradin system of cutaneeous changes in onchocerciasis. Br. J. Dermatol. 129, 260-269.

Murdoch, M.E., Payton, A., Abiose, A., Thomson, W., Panicker, V.K., Dyer, P.A., Jones, B.R., Maizels, R.M., Ollier, W.E. (1997). HLA-DQ alleles associate with cutaneous features of onchocerciasis. The Kaduna-London-Manchester Collaboration for Research on Onchocerciasis. Hum Immunol. 55:46-52.

Nielsen, R. (2005). Molecular signatures of natural selection. Ann. Rev. Genet. 39, 197–218.

Noma, M., Nwoke, B.E., Nutall, I., Tambala, P.A, Enyong, P., Namsenmo, A., Remme, J., Amazigo, U.V., Kale, O.O., and Seketeli, A. (2002). Rapid epidemiological mapping Of Onchocerciasis (REMO): its application by the African Programme for Onchocerciasis Control (APOC). Ann. Trop. Med. Parasitol. 96, 29-39.

Ottesen, E.A., Duke, B.O., Karam, M., and Behbehani, K. (1997). Strategies and tools for the control/elimation of lymphatic filariasis. Bull World Health Organ. 75, 491-503.

Pearlman, E., and Hall, L.R. (2000). Immune mechanisms in Onchocerca volvulus-mediated corneal disease (river blindness). Parasite Immunol. 22, 625-631.

Prod'hon, J., Boussinesq, M., Fobi, G., Prud'hom, J.M., Enyong, P., Lafleur, C., Quillévéré, D. (1991). Control of onchocerciasis with ivermectin: results of a mass campaign in northern Cameroon. Bull World Health Organ. 69:443-50.

Remme, J., Baker, R.H., De Sole, G., Dadzie, K.Y., Walsh, J.F., Adams, M.A., Alley, E.S., Avissey, H.S.(1989). A community trial of ivermectin in the onchocerciasis focus of Asubende, Ghana. I. Effect on the microfilarial reservoir and the transmission of Onchocerca volvulus. Trop Med Parasitol. 40:367-74

Rickards., O., Tartaglia, M., Martinez Labarga, C., and De Stefano, G.F., (1994). Genetic characterization of the Cayapa Indians of Ecuador and their genetic relationships with other native american population. Hum. Biol. 66, 299-322.

Robinson, J., Malik, A., Parham, P., Bodmer, J.G., Marsh, S.G.E. (2000). IMGT/HLA - a sequence database for the human major histocompatibility complex. Tissue Antigens. *55*:280-287.

Rougemont, A., Thylefors, B., Ducam, M., Prost, A., Ranque, P., Delmont, J.(1980) Treatment of onchocerciasis in hyperendemic communities in West Africa with small, gradually increasing doses of suramin. 1. Parasitological results and ophthalmological surveillance in a region where transmission has not been interrupted. Bull World Health Organ.58:917-22.

Rougemont, A., Hien, M., Thylefors, B., Prost, A., Rolland, A. (1984). Treatment of onchocerciasis with low, increasing doses of suramin in hyperendemic communities of Western Africa: 2. Clinical parasitologic and ophthalmologic results in a zone where transmission is controlled. Bull World Health Organ. 62:261-9.

Romero-Sandoval, N.C. (2007); Informacin epidemiolgica provincial de santo domingo de los tschilas aos 2007 2008 y enero marzo 2009.pdf, www.msp.gov.ec.

Saiki, R.K., Schark S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis of sicule cell anemia. Science 230, 1350-1354.

Saint Andre, A., Blackwell, N.M., Hall, L.R., Hoerauf, A., Bratting, N.W., Volkmann, L., Taylor, M.J., Ford, L., Hise, A.G., Lass, J.H., Diaconu, E., and Pearlman, E. (2002). The role of endosymbiontic Wolbachia bacteria in the pathogenesis of river blindness. Science 295, 1892-1895.

Sambrook, J., Fritsch, E.F., and Maniatis, T.; Hrsg. (1989). Molecular Cloning - A Laboratory Manual, 2nd Edition. (Cold Spring Habour Laboratory Press, New York).

Scozzari, R., Torroni, A., Semino, O., Cruciani, F., Spedini, G., Santachiara Benerecetti, S.A. (1994). Genetic studies in Cameroon: mitochondrial DNA polymorphisms in Bamileke. Hum Biol. 66:1-12.

Simon, C., Franke, A., and Martin, A.(1991). The polymerase chain reaction: DNA extraction and amplification. In: Molecular taxonomy, Hewitt, G.M., Johnston, A.W.B., and Young, J.P.W. NATO Advance Studies Institutes.

Simons, M.J., Limm, T.M., Naughton, M.J., Quinn, D.L., McGinnis, M.D., and Ashdown, M.L. (1993). Strategy for definition of DR/DQ haplotypes in the 4AOHW cell panel using noncoding sequence polymorphisms. Hum. Immunol. 38, 69-74.

Spedini, G., Destro-Bisol, G., Mondovi`, S., Kaptue´, L., Taglioli, L., Paoli, G.(1999). The Peopling of Sub-Saharan Africa: The Case Study of Cameroon. Am. J. Phys. Anthropol. 110:143–162.

Stein, S., Drabbels, J., van 't Sant, P., Witvliet, M.D., Bein, G., Claas, F.H., Doxiadis, I., Vox, S. (1996) The use of DNA typing for human platelet-specific antigens in the daily routine: a case report. 71:131-137.

Stephens, M., Smith, N.J., and Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. Am. J. Hum.Gen. 68, 978-989.

Taylor, M.J., Hoerauf, A. (1999) Wolbachia bacteria of filarial nematodes. Parasitol Today. 15:437-42.

Taylor, M.J., Bandi, C., and Hoerauf, A. (2005). Wolbachia bacterial endosymbionts of filarial nematodes. Adv. Parasitol. 60, 245-284.

The International HapMap Consortium (2005). A haplotype map of the human genome. Nature 437, 1299-1320.

Thein, S.L., Wallace, R.B., in: Human Genetic Diseases—A Practical Approach, IRL Press, Oxford, 1986, pp. 33–50.

Thylefors, B., Négrel, A.D, Pararajasegaram, R., Dadzie, K.Y. (1995) Global data on blindness. Bull World Health Organ.73(1):115-21.

Vejbaesya, S., Chierakul, N., Luangtrakool, K., Srinak, D., Stephens, H.A. (2002). Associations of HLA class II alleles with pulmonary tuberculosis in Thais. Eur J Immunogenet. 29:431-4.

van Dijk, A., Melchers, R., Tilanus, M. (2007). HLA-DQB1 sequencing-based typing updated.Tissue Antigens: 69: 64–5.

Walsh, E.C., Mather, K.A., Schaffner, S.F., Farwell, L., Daly, M.J., Patterson, N., Cullen, M., Carrington, M., Bugawan, T.L., Erlich, H., Campbell, J., Barrett, J., Miller, K., Thomson, G., Lander, E.S., Rioux, J.D. (2003). An integrated haplotype map of the human major histocompatibility complex. Am. J. Hum. Genet. 73, 580-90.

Wakelin, S. (1988) Views on health visiting the elderly. Health Visit. 1988 Jan;61(1):23.

Wassom, D.L., Krco, C.J., and David, C.S. (1987). IgE expression and susceptibility to parasitic infection. Immunol. Today 8, 39-43.

WHO (1987). Protective immunity and vaccination in onchocerciasis and lymphatic filariasis. Report of the 13th Scientific working group on filariasi. Geneva: WHO document TDR/ FIL/ SWG 13, 1-167.

WHO (1994). The onchocerciasis control program in West Africa. Public Health in action 5, 1-20.

WHO (1995). Onchocerciasis and its control. Report of a WHO Expert Committee on Onchocerciasis Control. World Health Organ. Tech. Rep. Ser. 852, 1-104.

WHO Report: (2006) A strategic overview of the future of onchocerciasis control in Africa.

Whitworth, J.A., Gilbert, C.E., Mabey, D.M., Maude, G.H., Morgan, D., Taylor, D.W. (1991). Effects of repeated doses of ivermectin on ocular onchocerciasis: community-based trial in Sierra Leone. Lancet. 2:1100-3.

Woodruff, A.W., Choycw, D.P., Muci-Mendoza, F., Hills, M., and Pettit, L.E. (1966). Onchocerciasis in Guatemala. A clinical and parasitological study with comparisons between the disease there and in East Africa. Trans. R. Soc. Trop. Med. Hyg. 60, 707-19.

7. APPENDIX 1

DNA EXTRACTION FROM PLASMA:

1. Shake the plasma for 20 sec. and place in a 1-ml ultracentrifuge tube (Beckman Instruments).
2. Add 3 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl).
3. Shake 20 sec. and place the tubes in ultracentrifuge using the rotor 70.1 (Beckman Instruments).
4. Cold centrifuge for 3 hours at 40,000 rpm.
5. Discard the supernatant and ensure that the pellet remains on the wall.
6. Resuspend the pellet in 200 µl of solution consisting of proteinase K (Roche Diagnostics GmbH, Germany) and 0.1% SDS.
7. Shake 20 sec. and centrifuge for a few seconds.
8. Take the liquid from the tubes and place in a 1.5 ml Eppendorf.
9. Incubate at 65°C for 48 hours.
10. Add 400 µl of phenol-chloroform-isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1mM EDTA (Sigma) and stir until it becomes cloudy.
11. Shake 10 sec. and centrifuge at 13,000 rpm for 3 minutes.
12. Add 100 µl of ddH_2O to a Microcon 100 (Millipore Corporation, Bedford USA) and subsequently transfer the aqueous phase to the Eppendorf.
13. Centrifuge at 2,500 rpm for 25 minutes.
14. Discard the filtrate and add 200 µl of sterile H_2O and spin at 2,500 rpm for 25 minutes.
15. Place the filter into a new Eppendorf, add 60 µl of ddH_2O into the filter, and resuspend the DNA.
16. Invert the filter and centrifuge at 3,500 rpm for 5 minutes to collect DNA.

DNA EXTRACTION FROM COAGULATE

1. Thaw the coagulate, then shake and centrifuge.
2. Add 300 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0 and 2% SDS) to a 1.5 ml Eppendorf tube.
3. Add 300 µl of sample.
4. Add 7.5 µl of proteinase K (Roche Diagnostics GmbH, Germany) (20mg/ml).
5. Incubate at 56°C overnight.
6. Shake and centrifuge.
7. Add 400 µl of phenol / chloroform / isoamyl alcohol (25:24:1) saturated with 10 mM Tris pH 8.0, 1 mM EDTA.
8. Stir until you get a cloudy emulsion.
9. Centrifuge at 13,000 rpm for 3 minutes.
10. Add 100 µl of sterile ddH_2O in a tube Microcon 100 and transfer the aqueous phase from the Eppendorf tube to the Microcon 100.
11. Centrifuge at 2,500 rpm for 25 minutes.
12. Discard the liquid and add 200 µl of ddH_2O.
13. Centrifuge at 2,500 rpm for 25 minutes.
14. Place the filter into a new Eppendorf tube and add 100 µl of ddH_2O.
15. Gently invert the filter and centrifuge at 2,500 rpm for 5 minutes to collect DNA.

PURIFICATION OF AMPLIFIED SAMPLES

1. Bring the product to 50 µl with autoclaved /sterile bi-distilled H_2O and add 5 µl of sodium acetate 3M pH 4,6 and 200 µl of cold absolute ethanol.
2. Stir and allow to precipitate amplified for 20 minutes at -20°C. Separate the amplified product from the rest of the mixture by centrifugation at 14,000 rpm for 20 minutes.
3. Draw out the liquid phase.
4. Wash the pellet by adding 200 µl of 70% cold ethanol and make a second centrifugation at 14,000 rpm for 20 minutes.

5. Discard the liquid and dry the obtained pellet to gain the amplified and purified product.

PURIFICATION OF DYED SAMPLES

1.Add to the sample 40 µl of ddH_2O, 5 µl of sodium acetate 3M pH 4.6, and 200 µl of cold ethanol.

2.Leave at room temperature for 15 minutes.

3.Centrifuge samples at 2,500 rpm for 30 minutes.

4.Aspirate the supernatant.

5.Perform a wash with 200 µl of 80% ethanol.

6.Centrifuge at 14,000 rpm for 10 minutes.

7.Discard the supernatant again.

8.Dry for 3-4 minutes